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(54) Title: METHOD FOR DETECTION OF DRUG-INDUCED MUTATIONS IN THE REVERSE TRANSCRIPTASE GENE

(57) Abstract

The present invention relates to a method for the rapid and reliable detection of drug-induced mutations in the reverse transcriptase gene allowing the simultaneous characterization of a range of codons involved in drug resistance using specific sets of probes optimized to function together in a reverse-hybridization assay. More particularly, the present invention relates to a method for determining the susceptibility to antiviral drugs of HIV strains present in a biological sample, comprising: (i) if need be releasing, isolating or concentrating the polynucleic acids present in the sample; (ii) if need be amplifying the relevant part of the reverse transcriptase genes present in said sample with at least one suitable primer pair; (iii) hybridizing the polynucleic acids of step (i) or (ii) with at least two RT gene probes hybridizing specifically to one or more target sequences with said probes being applied to known locations on a solid support and with said probes being capable of simultaneously hybridizing to their respective target regions under appropriate hybridization and wash conditions allowing the detection of homologous targets, or said probes hybridizing specifically with a sequence complementary to any of said target sequences, or a sequence wherein T is replaced by U; (iv) detecting the hybrids formed in step (iii); (v) inferring the nucleotide sequence at the codons of interest and/or the amino acids of the codons of interest and/or antiviral drug resistance spectrum, and possibly the type of HIV isolates involved from the differential hybridization signal(s) obtained in step (iv).

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Method for detection of drug-induced mutations in the reverse transcriptase gene.

The present invention relates to the field of HIV diagnosis. More particularly, the present invention relates to the field of diagnosing the susceptibility of an HIV sample to antiviral drugs used to treat HIV infection.

The present invention relates to a method for the rapid and reliable detection of drug-induced mutations in the HIV reverse transcriptase gene allowing the simultaneous characterization of a range of codons involved in drug resistance using specific sets of probes optimized to function together in a reverse-hybridisation assay.

During the treatment of human immunodeficiency virus (HIV) type 1 infected individuals with antiretroviral nucleoside analogs emergence of resistance against these drugs has been observed. The mechanism responsible for the resistance is not fully understood, since the appearance of a resistant virus in not always correlated with clinical deterioration (Boucher et al. 1992). Amongst the reverse transcriptase (RT) inhibitors, the nucleoside analogs 3'-azido-2',3'-dideoxyThymidine (AZT, Zidovudine), 2',3'-dideoxyInosine (ddI), 2',3'-dideoxyCytidine (ddC), (-)-β-L-2',3'-dideoxy-3'-thioCytidine (3TC), 2',3'-didehydro-3'deoxyThymidine (D4T) and (-)-2'3'-dideoxy-5-fluoro-3'-thiacytidine (FTC) are the most important, since they show a favourable ratio of toxicity for the host versus efficacy as antiviral. All these compounds act in a similar way, namely they serve, after intracellular phosphorylation, as chain terminators of the RT reaction. Upon prolonged treatment with these nucleoside analogs, accumulation of mutations in the viral reverse transcriptase gene (RT) occur, thereby escaping the inhibitory effect of the antivirals. The most important mutations induced by the above compounds and leading to gradually increasing resistance were found at amino acid (aa) positions 41 (M to L), 69 (T to D), 70 (K to R), 74 (L to V), 181 (Y to C), 184 (M to V) and 215 (T to Y or F) (Schinazi et al., 1994). Mutations at aa 65, 67, 75 and 219 have also been reported but these were only showing a minor decrease in sensitivity. More recently, multi-drug-resistant HIV-1 strains were described showing aa changes at codon 62, 75, 77, 116, and 151 (Iversen et al., 1996). In general, these aa changes are the consequence of single point mutations at the first or second codon letter, but in the case of T69D (ACT to GAT), T215Y (ACC to TAC) and T215F (ACC to TTC), two nucleotide mutations are

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necessarry. Whether in these cases the single nucleotide mutation intermediates exist, and if they show any importance in the mechanism for acquiring resistance is as yet not reported. Third letter variations are in general not leading to an amino acid change, and are therefore seen as natural polymorphisms.

The regime for an efficient antiviral treatment is not clear at all. The appearance of one or several of these mutations during antiviral treatment need to be interpreted in conjunction with the virus load and the amount of CD4 cells. Indeed, since it has been shown that the effect of AZT resistance mutations can be suppressed after the appeareance of the 3TC induced M184V mutation, it is clear that disease progression is multifactorial. The influence of other simultaneous occuring mutations under different combination therapies with respect to the outcome and resistance of the virus has not yet been analysed systematically. In order to get a better insight into the mechanisms of resistance and HIV biology, it is necessarry to analyse follow-up plasma samples of antiviral treated patients for these mutational events together with the simultaneous occuring changes of virus titre and CD4 cells.

It is an aim of the present invention to develop a rapid and reliable detection method for determination of the antiviral drug resistance of viruses which contain reverse transcriptase genes such as HIV retroviruses and Hepadnaviridae present in a biological sample.

More particularly it is an aim of the present invention to provide a genotyping assay allowing the detection of the different HIV RT gene wild type and mutation codons involved in the antiviral resistance in one single experiment.

It is also an aim of the present invention to provide an HIV RT genotyping assay or method which allows to infer the nucleotide sequence at codons of interest and/or the amino acids at the codons of interest and/or the antiviral drug resistance spectrum, and possibly also infer the HIV type or subtype isolate involved.

Even more particularly it is an aim of the present invention to provide a genotyping assay allowing the detection of the different HIV RT gene polymorphisms representing wild-type and mutation codons in one single experimental setup.

It is another aim of the present invention to select particular probes able to discriminate wild-type HIV RT sequences from mutated or polymorphic HIV RT sequences conferring resistance to one or more antiviral drugs, such as AZT, ddI, ddC,

3TC or FTC, D4T or others.

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV RT sequences from mutated or polymorphic HIV RT sequences conferring resistance to AZT.

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV RT sequences from mutated HIV RT sequences conferring resistance to ddI.

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV RT sequences from mutated HIV RT sequences conferring resistance to ddC.

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV RT sequences from mutated HIV RT sequences conferring resistance to 3TC.

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV RT sequences from mutated HIV RT sequences conferring resistance to D4T.

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV RT sequences from mutated HIV RT sequences conferring resistance to FTC.

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV RT sequences from mutated HIV RT sequences conferring resistance to multiple nucleoside analogues (i.e. multidrug resistance).

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV RT sequences from mutated HIV RT sequences conferring resistance to nevirapine.

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV RT from mutated HIV RT sequences involving at least one of amino acid positions 41 (M to L), 50 (I to T), 67 (D to N). 69 (T to D), 70 (K to R), 74 (L to V), 75 (V to T), 151 (Q to M or L), 181 (Y to C), 184 (M to V), 215 (T to Y or F) and 219 (K to Q or E) of the viral reverse transcriptase (RT) gene.

It is particularly an aim of the present invention to select a particular set of probes, able to discriminate wild-type HIV RT sequences from mutated HIV RT sequences

conferring resistance to any of the antiviral drugs defined above with this particular set of probes being used in a reverse hybridisation assay.

It is moreover an aim of the present invention to combine a set of selected probes able to discriminate wild-type HIV RT sequences from mutated HIV RT sequences conferring resistance to antiviral drugs with another set of selected probes able to identify the HIV isolate, type or subtype present in the biological sample, whereby all probes can be used under the same hybridisation and wash-conditions.

It is also an aim of the present invention to select primers enabling the amplification of the gene fragment(s) determining the antiviral drug resistance trait of interest.

The present invention also aims at diagnostic kits comprising said probes useful for developing such a genotyping assay.

All the aims of the present invention have been met by the following specific embodiments.

The present invention relates more particularly to a method for determining the susceptibility to antiviral drugs of an HIV retrovirus present in a biological sample, comprising:

(i) if need be releasing, isolating or concentrating the polynucleic acids present in the sample;

(ii) if need be amplifying the relevant part of the reverse transcriptase genes present in said sample with at least one suitable primer pair;

(iii) hybridizing the polynucleic acids of step (i) or (ii) with at least two RT gene probes hybridizing specifically to at least one target sequence as mentioned in any of figure 1and tables 1, 2 or 4, with said probes being applied to known locations of a solid support and with said probes being capable of simultaneously hybridizing to their respective target regions under appropiate hybridization and wash conditions allowing the detection of homologous targets, or with said probes hybridizing specifically with a sequence complementary to any of said target sequences, or a sequence wherein T in said target sequence is replaced by U;

(iv) detecting the hybrids formed in step (iii);

(v) and in most cases, inferring the nucleotide sequence at the codons of interest and/or the amino acids at the codons of interest and/or the antiviral drug resistance

spectrum, and possibly the type of HIV isolates involved from the differential hybridization signal(s) obtained in step (iv).

The relevant part of the RT gene refers to the regions in the RT gene harboring mutations causing resistance to antiviral drugs as described above and is particularly comprised between codons 1 and 241, and more particularly between codons 29 and 220 of the RT gene.

According to a preferred embodiment of the present invention, step (iii) is performed using a set of at least 2, preferably at least 3, more preferably at least 4 and most preferably at least 5 probes meticulously designed as such that they show the desired hybridization results, when used in a reverse hybridisation assay format, more particularly under the same hybridization and wash conditions.

According to a preferred embodiment, the present invention relates to a set of at least 2 probes each targetting one or more of the nucleoside RT inhibitor induced nucleotide changes or target sequences including such a nucleotide change as indicated in any of Figure 1 or Tables 1, 2 or 4. The numbering of HIV-1 RT gene encoded amino acids is as generally accepted in literature.

More prefererably, the present invention relates to a set of two or more probes each targetting two, three, four, five or more different nucleoside RT inhibitor induced nucleotide changes as indicated in any of Figure 1or Tables 1, 2 or 4.

More particularly, the present invention relates to a set of at least 2 probes allowing the characterization of a wild-type, polymorphic or mutated codon at any one of the drug-induced mutation positions represented in any of Figure 1 or Tables 1 or 2 or at any one of the polymorphic positions represented in Table 4.

Even more particularly, the present invention relates to a set of at least 2 probes allowing the characterization of a wild-type, polymorphic or mutated codon at any of the positions represented in Figure 1.

All the above mentioned sets of probes have as a common characteristic that all the probes in said set are designed so that they can function together in a reverse-hybridization assay, more particularly under similar hybridization and wash conditions.

A particularly preferred set of probes selected out of the probes with SEQ ID NO 1 to 161 of Table 3 is described in example 2.2 and is indicated in Table 4 and Figure 2. The particularly selected probes are also indicated in Table 3.

A particularly preferred embodiment of the present invention is a method for determining the susceptibility to antiviral drugs of an HIV isolates in a sample using a set of probes as defined above, wherein said set of probes is characterized as being chosen such that for a given mutation disclosed in any of Figure 1, or Tables 1, 2 or 4 the following probes are included in said set :

- at least one probe for detecting the presence of drug induced mutation at said position;
- at least one probe for detecting the presence of a wild-type sequence at said position;
- preferably also (an) additional probe(s) for detecting wild-type polymorphisms at positions surrounding the mutation position.

Inclusion of the latter two types of probes greatly contributes to increasing the sensitivity of said assays as demonstrated in the examples section.

A particularly preferred set of probes in this respect is shown in Tables 3 and 4 and Figures 2 and 3.

Selected sets of probes according to the present invention include at least one probe, preferably at least two probes, characterizing the presence of a drug-induced mutation in a codon position chosen from the following list of codons susceptible to mutations in the HIV RT gene: 41, 50, 67, 69, 70, 74, 75, 151, 181, 184, 215 or 219. Said probes being characterized in that they can function in a method as set out above.

Also selected probes according to the present invention are probes which allow to differentiate any of the nucleotide changes as represented in any of Figure 1 or Tables 1, 2 or 4. Said probes being characterized in that they can function in a method as set out above.

Also selected sets of probes for use in a method according to the present invention include at least one, preferably at least two (sets of) probes, with said probes characterizing the presence of a drug-induced mutation in two codon positions chosen from the following list of codon combinations, with said codons being susceptible to mutations in the HIV RT gene: 41 and/or 50; 41 and/or 67; 41 and/or 69; 41 and/or 70; 41 and/or 74; 41 and/or 75; 41 and/or 151; 1 and/or 181; 41 and/or 184; 41 and/or 215; 41 and/or 219; 50 and/or 67; 50 and/or 69; 50 and/or 70; 50 and/or 74; 50 and/or 75; 50 and/or 75; 50 and/or 151; 50 and/or 181; 50 and/or 184; 50 and/or 215; 50 and/or 219; 67 and/or

69; 67 and/or 70; 67 and/or 74; 67 and/or 75; 67 and/or 151; 67 and/or 181; 67 and/or 184; 67 and/or 215; 67 and/or 219; 69 and/or 70; 69 and/or 74; 69 and/or 75; 69 and/or 151; 69 and/or 181; 69 and/or 184; 69 and/or 215; 69 and/or 219; 70 and/or 74; 70 and/or 75; 70 and/or 151; 70 and/or 181; 70 and/or 184; 70 and/or 215; 70 and/or 219; 74 and/or 75; 74 and/or 151; 74 and/or 181; 74 and/or 184; 74 and/or 215; 74 and/or 219; 75 and/or 151; 75 and/or 181; 75 and/or 184; 75 and/or 215; 75 and/or 219; 151 and/or 181; 151 and/or 184; 151 and/or 215; 151 and/or 219; 181 and/or 184; 181 and/or 215; 181 and/or 219; 184 and/or 215; 184 and/or 219; 215 and/or 219.

Even more preferred selected sets of probes for use in a method according to the present invention include in addition to the probes defined above a third (set of) probe(s) characterizing the presence of a third drug-induced mutation at any of positions 41, 50, 67, 69, 70, 74, 75, 151, 181, 184, 215 or 219, or particular combinations thereof.

Particularly preferred is also a set of probes which allows simultaneous detection of antiviral resistance at codons 41, 50, 67, 69, 70, 74, 75, 151, 181, 184 and 215, possibly also at codon 219.

An additional embodiment of the present invention includes at least one probe, preferably at least two probes, characterizing the presence of a drug-induced mutation in codon positions chosen from the list of codons susceptible to mutations in the HIV RT gene as mentioned in any of Table 1 or 2, such as at codons 65, 115, 150, 98, 100, 103, 106, 108, 188, 190, 138, 199, 101, 179, 236, 238 or 233, with said probes forming possibly part of a composition.

Particularly preferred embodiments of the invention thus include a set of probes for codon 41 comprising at least one, preferably at least two, probe(s) for targetting at least one, preferably at least two, nucleotide changes in any the following codons as represented in region I in Figure 1:
wild-type codon E40 (GAA) or polymorphic codon E40 (GAG), mutant codon L41 (TTG) or L41 (CTG) or wild-type codon M41 (ATG), wild-type codon E42 (GAA) or polymorphic codon E42 (GAG), wild-type codon K43 (AAG) or polymorphic codon K43 (AAA) or polymorphic E43 (GAA).

Particularly preferred embodiments of the invention thus include a set of probes for codon 50 comprising at least one, preferably at least two, probe(s) for targetting at least one, preferably at least two, nucleotide changes in any the following codons as represented

in region II in Figure 1:
wild-type codon K49 (AAA) or polymorphic codon R49 (AGA), mutant codons V50 (GTT) or T50 (ACG), wild-type codon I50 (ATT) or polymorphic codon I50 (ATC).

Particularly preferred embodiments of the invention thus include a set of probes for codons 67-70 comprising at least one, preferably at least two, probe(s) for targetting at least one, preferably at least two, nucleotide changes in any of the following codons as represented in region III in Figure 1:
wild-type K65 (AAA) or polymorphic K65 (AAG), wild-type K66 (AAA) or polymorphic K66 (AAG), wild-type D67 (GAC) or mutant N67 (AAC), wild-type T69 (ACT) or polymorphic T69 (ACA), mutant D69 (GAT) or N69 (AAT) or A69 (GCT), wild-type K70 (AAA), polymorphic K70 (AAG) or mutant R70 (AGA).

Particularly preferred embodiments of the present invention include a set of probes for codons 74-75 comprising at least one, preferably at least two, probes for targetting at least one, preferably at least two, nucleotide changes in any of the following codons as represented in region IV of Figure 1:
wild-type K73 (AAA) or polymorphic K73 (AAG), wild-type L74 (TTA) or mutant V74 (GTA), wild-type V75 (GTA) or polymorphic V75 (GTG) or mutant T75 (ACA), wild-type D76 (GAT) or polymorphic D76 (GAC).

Particularly preferred embodiments of the present invention include a set of probes for codon 151 comprising at least one, preferably at least two, probes for targetting at least one, preferably at least two, nucleotide changes in any of the following codons as represented in region V of Figure 1:
wild-type L149 (CTT) or polymorphic L149 (CTC) or L149 (CTG), wild-type P150 (CCA) or polymorphic P150 (CCG), wild-type Q151 (CAG) or mutant M151 (ATG) or L151 (CTG) or polymorphic Q151 (CAA).

Particularly preferred embodiments of the present invention include a set of probes for codon 181-184 comprising at least one, preferably at least two, probe(s) for targetting at least one, preferably at least two, nucleotide changes in any of the following codons as represented in region VI of Figure 1:
wild-type Y181 (TAT) or mutant C181 (TGT), wild-type Q182 (CAA) or polymorphic Q182 (CAG), wild-type Y183 (TAC) or polymorphic Y183 (TAT), wild-type M184 (ATG) or mutant V184 (GTG) or I184 (ATA) or G184 (AGG), wild-type D185 (GAT) or

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polymorphic D185 (GAC), wild-type D186 (GAT) or polymorphic E186 (GAG), wild-type L187 (TTA) or polymorphic G187 (GGA) or V187 (GTA).

Particularly preferred embodiments of the present invention include a set of probes for codon 215 comprising at least one, preferably at least two, probe(s) for targetting at least one, preferably at least two, nucleotide changes in any of the following codons as represented in region VII of Figure 1 :
wild-type G213 (GGA) or polymorphic G213 (GGG), wild-type F214 (TTT) or polymorphic F214 (TTC) or L214 (CTT) or L214 (TTA), wild-type T215 (ACC) or polymorphic T215 (ACT), mutant Y215 (TAC) or F215 (TTC).

Particularly preferred embodiments of the present invention include a set of probes for codon 219 comprising at least one, preferably at least two, probe(s) for targetting at least one, preferably at least two, nucleotide changes in any of the following codons as represented in region VIII of Figure 1 :
wild-type D218 (GAC) or polymorphic D218 (GAT), wild-type K219 (AAA) or polymorphic K219 (AAG) or mutant Q219 (CAA) or E219 (GAA), wild-type K220 (AAA) or polymorphic K220 (AAG).

Examples of probes of the invention are represented in Tables 3 and 4, and Figures 2 and 3. In Table 3, the probes withheld after selection are indicated using the letter "y". These probes of the invention are designed for attaining optimal performance under the same hybridization conditions so that they can be used in sets of at least 2 probes for simultaneous hybridization; this highly increases the usefulness of these probes and results in a significant gain in time and labour. Evidently, when other hybridization conditions would be preferred, all probes should be adapted accordingly by adding or deleting a number of nucleotides at their extremities. It should be understood that these concommitant adaptations should give rise to essentially the same result, namely that the respective probes still hybridize specifically with the defined target. Such adaptations might also be necessary if the amplified material should be RNA in nature and not DNA as in the case for the NASBA (nucleic acid sequence-based amplification) system.

The selection of the preferred probes of the present invention is based on a reverse hybridization assay using immobilized oligonucleotide probes present at distinct locations on a solid support. More particularly the selection of preferred probes of the present invention is based on the use of the Line Probe Assay (LiPA) principle which is a reverse

hybridization assay using oligonucleotide probes immobilized as parallel lines on a solid support strip (Stuyver et al. 1993; international application WO 94/12670). This approach is particularly advantageous since it is fast and simple to perform. The reverse hybridization format and more particularly the LiPA approach has many practical advantages as compared to other DNA techniques or hybridization formats, especially when the use of a combination of probes is preferable or unavoidable to obtain the relevant information sought.

It is to be understood, however, that any other type of hybridization assay or format using any of the selected probes as described further in the invention, is also covered by the present invention.

The reverse hybridization approach implies that the probes are immobilized to certain locations on a solid support and that the target DNA is labelled in order to enable the detection of the hybrids formed.

Methods for detecting nucleotide changes in RT genes of other viruses which have been found to harbour a pattern of drug-resistance mutation similar to the one observed for HIV based on the same principles as set out in the present invention should be understood as also being covered by the scope of the present invention.

The following definitions serve to illustrate the terms and expressions used in the present invention.

The term "antiviral drugs" refers particularly to an antiviral nucleoside analog or any other RT inhibitor. Examples of such antiviral drugs and the mutation they may cause in the HIV-RT gene are disclosed in Schinazi et al., 1994 and Mellors et al., 1995. The contents of the latter two documents particularly are to be considered as forming part of the present invention. The most important antiviral drugs focussed at in the present invention are disclosed in Tables 1 to 2.

The term "drug-induced mutation" refers to a mutation in the HIV RT gene which provokes a reduced susceptibility of the isolate to the respective drug.

The target material in the samples to be analysed may either be DNA or RNA, e.g.: genomic DNA, messenger RNA, viral RNA or amplified versions thereof. These molecules are also termed polynucleic acids.

It is possible to use genomic DNA or RNA molecules from HIV samples in the methods according to the present invention.

Well-known extraction and purification procedures are available for the isolation of RNA or DNA from a sample (f.i. in Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbour Laboratory Press (1989)).

The term "probe" refers to single stranded sequence-specific oligonucleotides which have a sequence which is complementary to the target sequence to be detected.

The term "target sequence" as referred to in the present invention describes the nucleotide sequence of the wildtype, polymorphic or drug induced variant sequence of the RT gene to be specifically detected by a probe according to the present invention. This nucleotide sequence may encompass one or several nucleotide changes. Target sequences may refer to single nucleotide positions, codon positions, nucleotides encoding amino acids or to sequences spanning any of the foregoing nucleotide positions. In the present invention said target sequence often includes one or two variable nucleotide positions.
It is to be understood that the complement of said target sequence is also a suitable target sequence in some cases. The target sequences as defined in the present invention provide sequences which should be complementary to the central part of the probe which is designed to hybridize specifically to said target region.

The term "complementary" as used herein means that the sequence of the single stranded probe is exactly the (inverse) complement of the sequence of the single-stranded target, with the target being defined as the sequence where the mutation to be detected is located.

Since the current application requires the detection of single basepair mismatches, very stringent conditions for hybridization are required, allowing in principle only hybridization of exactly complementary sequences. However, variations are possible in the length of the probes (see below), and it should be noted that, since the central part of the probe is essential for its hybridization characteristics, possible deviations of the probe sequence versus the target sequence may be allowable towards head and tail of the probe, when longer probe sequences are used. These variations, which may be conceived from the common knowledge in the art, should however always be evaluated experimentally, in order to check if they result in equivalent hybridization characteristics than the exactly complementary probes.

Preferably, the probes of the invention are about 5 to 50 nucleotides long, more preferably from about 10 to 25 nucleotides. Particularly preferred lengths of probes include

10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 nucleotides. The nucleotides as used in the present invention may be ribonucleotides, deoxyribonucleotides and modified nucleotides such as inosine or nucleotides containing modified groups which do not essentially alter their hybridisation characteristics.

Probe sequences are represented throughout the specification as single stranded DNA oligonucleotides from the 5' to the 3' end. It is obvious to the man skilled in the art that any of the below-specified probes can be used as such, or in their complementary form, or in their RNA form (wherein T is replaced by U).

The probes according to the invention can be prepared by cloning of recombinant plasmids containing inserts including the corresponding nucleotide sequences, if need be by cleaving the latter out from the cloned plasmids upon using the adequate nucleases and recovering them, e.g. by fractionation according to molecular weight. The probes according to the present invention can also be synthesized chemically, for instance by the conventional phospho-triester method.

The term "solid support" can refer to any substrate to which an oligonucleotide probe can be coupled, provided that it retains its hybridization characteristics and provided that the background level of hybridization remains low. Usually the solid substrate will be a microtiter plate, a membrane (e.g. nylon or nitrocellulose) or a microsphere (bead) or a chip. Prior to application to the membrane or fixation it may be convenient to modify the nucleic acid probe in order to facilitate fixation or improve the hybridization efficiency. Such modifications may encompass homopolymer tailing, coupling with different reactive groups such as aliphatic groups, NH_2 groups, SH groups, carboxylic groups, or coupling with biotin, haptens or proteins.

The term "labelled" refers to the use of labelled nucleic acids. Labelling may be carried out by the use of labelled nucleotides incorporated during the polymerase step of the amplification such as illustrated by Saiki et al. (1988) or Bej et al. (1990) or labelled primers. or by any other method known to the person skilled in the art. The nature of the label may be isotopic (^{32}P, ^{35}S, etc.) or non-isotopic (biotin, digoxigenin, etc.).

The term "primer" refers to a single stranded oligonucleotide sequence capable of acting as a point of initiation for synthesis of a primer extension product which is complementary to the nucleic acid strand to be copied. The length and the sequence of the primer must be such that they allow to prime the synthesis of the extension products.

Preferably the primer is about 5-50 nucleotides long. Specific length and sequence will depend on the complexity of the required DNA or RNA targets, as well as on the conditions of primer use such as temperature and ionic strenght.

The fact that amplification primers do not have to match exactly with the corresponding template sequence to warrant proper amplification is amply documented in the literature (Kwok et al., 1990).

The amplification method used can be either polymerase chain reaction (PCR; Saiki et al., 1988), ligase chain reaction (LCR; Landgren et al., 1988; Wu & Wallace, 1989; Barany, 1991), nucleic acid sequence-based amplification (NASBA; Guatelli et al., 1990; Compton, 1991), transcription-based amplification system (TAS; Kwoh et al., 1989), strand displacement amplification (SDA; Duck, 1990; Walker et al., 1992) or amplification by means of Qß replicase (Lizardi et al., 1988; Lomeli et al., 1989) or any other suitable method to amplify nucleic acid molecules known in the art.

The oligonucleotides used as primers or probes may also comprise nucleotide analogues such as phosphorothiates (Matsukura et al., 1987), alkylphosphorothiates (Miller et al., 1979) or peptide nucleic acids (Nielsen et al., 1991; Nielsen et al., 1993) or may contain intercalating agents (Asseline et al., 1984).

As most other variations or modifications introduced into the original DNA sequences of the invention these variations will necessitate adaptions with respect to the conditions under which the oligonucleotide should be used to obtain the required specificity and sensitivity. However the eventual results of hybridisation will be essentially the same as those obtained with the unmodified oligonucleotides.

The introduction of these modifications may be advantageous in order to positively influence characteristics such as hybridization kinetics, reversibility of the hybrid-formation, biological stability of the oligonucleotide molecules, etc.

The "sample" may be any biological material taken either directly from the infected human being (or animal), or after culturing (enrichment). Biological material may be e.g. expectorations of any kind, broncheolavages, blood, skin tissue. biopsies, sperm, lymphocyte blood culture material, colonies, liquid cultures, faecal samples, urine etc.

The sets of probes of the present invention will include at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more probes. Said probes may be applied in two or more distinct and known positions on a

solid substrate. Often it is preferable to apply two or more probes together in one and the same position of said solid support.

For designing probes with desired characteristics, the following useful guidelines known to the person skilled in the art can be applied.

Because the extent and specificity of hybridization reactions such as those described herein are affected by a number of factors, manipulation of one or more of those factors will determine the exact sensitivity and specificity of a particular probe, whether perfectly complementary to its target or not. The importance and effect of various assay conditions, explained further herein, are known to those skilled in the art.

The stability of the [probe : target] nucleic acid hybrid should be chosen to be compatible with the assay conditions. This may be accomplished by avoiding long AT-rich sequences, by terminating the hybrids with G:C base pairs, and by designing the probe with an appropriate Tm. The beginning and end points of the probe should be chosen so that the length and %GC result in a Tm about 2-10°C higher than the temperature at which the final assay will be performed. The base composition of the probe is significant because G-C base pairs exhibit greater thermal stability as compared to A-T base pairs due to additional hydrogen bonding. Thus, hybridization involving complementary nucleic acids of higher G-C content will be stable at higher temperatures.

Conditions such as ionic strenght and incubation temperature under which a probe will be used should also be taken into account when designing a probe. It is known that hybridization will increase as the ionic strenght of the reaction mixture increases, and that the thermal stability of the hybrids will increase with increasing ionic strenght. On the other hand, chemical reagents, such as formamide, urea, DMSO and alcohols, which disrupt hydrogen bonds, will increase the stringency of hybridization. Destabilization of the hydrogen bonds by such reagents can greatly reduce the Tm. In general, optimal hybridization for synthetic oligonucleotide probes of about 10-50 bases in length occurs approximately 5°C below the melting temperature for a given duplex. Incubation at temperatures below the optimum may allow mismatched base sequences to hybridize and can therefore result in reduced specificity.

It is desirable to have probes which hybridize only under conditions of high stringency. Under high stringency conditions only highly complementary nucleic acid hybrids will form: hybrids without a sufficient degree of complementarity will not form.

Accordingly, the stringency of the assay conditions determines the amount of complementarity needed between two nucleic acid strands forming a hybrid. The degree of stringency is chosen such as to maximize the difference in stability between the hybrid formed with the target and the nontarget nucleic acid. In the present case, single base pair changes need to be detected, which requires conditions of very high stringency.

The length of the target nucleic acid sequence and, accordingly, the length of the probe sequence can also be important. In some cases, there may be several sequences from a particular region, varying in location and length, which will yield probes with the desired hybridization characteristics. In other cases, one sequence may be significantly better than another which differs merely by a single base. While it is possible for nucleic acids that are not perfectly complementary to hybridize, the longest stretch of perfectly complementary base sequence will normally primarily determine hybrid stability. While oligonucleotide probes of different lengths and base composition may be used, preferred oligonucleotide probes of this invention are between about 5 to 50 (more particularely 10-25) bases in length and have a sufficient stretch in the sequence which is perfectly complementary to the target nucleic acid sequence.

Regions in the target DNA or RNA which are known to form strong internal structures inhibitory to hybridization are less preferred. Likewise, probes with extensive self-complementarity should be avoided. As explained above, hybridization is the association of two single strands of complementary nucleic acids to form a hydrogen bonded double strand. It is implicit that if one of the two strands is wholly or partially involved in a hybrid that it will be less able to participate in formation of a new hybrid. There can be intramolecular and intermolecular hybrids formed within the molecules of one type of probe if there is sufficient self complementarity. Such structures can be avoided through careful probe design. By designing a probe so that a substantial portion of the sequence of interest is single stranded, the rate and extent of hybridization may be greatly increased. Computer programs are available to search for this type of interaction. However, in certain instances, it may not be possible to avoid this type of interaction.

Standard hybridization and wash conditions are disclosed in the Materials & Methods section of the Examples. Other conditions are for instance 3X SSC (Sodium Saline Citrate), 20% deionized FA (Formamide) at 50°C.

Other solutions (SSPE (Sodium saline phosphate EDTA), TMACl (Tetramethyl

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ammonium Chloride), etc.) and temperatures can also be used provided that the specificity and sensitivity of the probes is maintained. If need be, slight modifications of the probes in length or in sequence have to be carried out to maintain the specificity and sensitivity required under the given circumstances.

In a more preferential embodiment, the above-mentioned polynucleic acids from step (i) or (ii) are hybridized with at least two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, twenty, or more of the above-mentioned target region specific probes, preferably with 5 or 6 probes, which, taken together, cover the "mutation region" of the RT gene.

The term "mutation region" means the region in the HIV RT gene sequence where most of the mutations responsible for antiviral drug resistance or other observed polymorphisms are located. A preferred part of this mutation region is represented in fig. 1. This mutation region can be divided into 8 important parts: drug induced variations and polymorphisms located within aa positions 38 to 44 of RT gene, drug induced variations and polymorphisms located within aa positions 47 to 53 of RT gene, drug induced variations and polymorphisms located within aa positions 65 to 72 of the RT gene, drug induced variations and polymorphisms located within aa positions 73 to 77 of the RT gene, drug-induced variations and polymorphisms located within aa positions 148 to 154 of the RT gene, drug-induced variations and polymorphisms located within aa positions 180 to 187 of the RT gene, drug induced variations and polymorphisms located within aa positions 212 to 216 of the RT gene and drug induced variations and polymorphisms located within aa positions 217 to 220 of the RT gene.

Since some mutations may be more frequently occurring than others, e.g. in certain geographic areas or in specific circumstances (e.g. rather closed communities) it may be appropiate to screen only for specific mutations, using a selected set of probes as indicated above. This would result in a more simple test, which would cover the needs under certain circumstances.

In order to detect the antiviral drug RT resistance pattern with the selected set of oligonucleotide probes, any hybridization method known in the art can be used (conventional dot-blot, Southern blot, sandwich, etc.).

However, in order to obtain fast and easy results if a multitude of probes are involved, a reverse hybridization format may be most convenient.

In a preferred embodiment the selected set of probes are immobilized to a solid support in known distinct locations (dots, lines or other figures). In another preferred embodiment the selected set of probes are immobilized to a membrane strip in a line fashion. Said probes may be immobilized individually or as mixtures to delineated locations on the solid support.

A specific and very user-friendly embodiment of the above-mentioned preferential method is the LiPA method, where the above-mentioned set of probes is immobilized in parallel lines on a membrane, as further described in the examples.

The invention also provides for any probes and primer sets designed to specifically detect or amplify specifically these RT gene polymorphisms, and any method or kits using said primer and probes sets.

The invention further provides for any of the probes as described above, as well as compositions comprising at least one of these probes.

The invention also provides for a set of primers allowing amplification of the mutation region of the RT gene in general.

Primers may be labeled with a label of choice (e.g. biotine). Different primer-based target amplification systems may be used, and preferably PCR-amplification, as set out in the examples. Single-round or nested PCR may be used.

The invention also provides for a kit for inferring the nucleotide sequence at codons of interest in the HIV RT gene and/or the amino acids corresponding to these codons and/or the antiviral drug resistance spectrum of HIV isolates present in a biological sample comprising the following components:

(i) when appropiate, a means for releasing, isolating or concentrating the polynucleic acids present in said sample;

(ii) when appropriate, at least one of the above-defined set of primers;

(iii) at least two of the probes as defined above, possibly fixed to a solid support;

(iv) a hybridization buffer, or components necessary for producing said buffer;

(v) a wash solution, or components necessary for producing said solution;

(vi) when appropriate, a means for detecting the hybrids resulting from the preceding hybridization.

(vii) when appropriate, a means for attaching said probe to a solid support.

The term "hybridization buffer" means a buffer enabling a hybridization reaction to

occur between the probes and the polynucleic acids present in the sample, or the amplified products, under the appropiate stringency conditions.

The term "wash solution" means a solution enabling washing of the hybrids formed under the appropiate stringency conditions.

A line probe assay (LiPA) was designed for the screening for variations at interesting amino acids in the HIV RT gene. The principle of the assay is based on reverse hybridization of an amplified polynucleic acid fragment such as a biotinylated PCR fragment of the HIV RT gene onto short oligonucleotides. The latter hybrid can then, via a biotine-streptavidine coupling, be detected with a non-radioactive colour developing system.

The present invention further relates to a reverse hybridization method wherein said oligonucleotide probes are immobilized, preferably on a membrane strip.

The present invention also relates to a composition comprising any of the probes as defined in Tables 3 and 4 or Figures 2 and 3.

The present invention relates also to a kit for inferring the HIV RT resistance spectrum of HIV in a biological sample, coupled to the identification of the HIV isolate involved, comprising the following components:

(i) when appropiate, a means for releasing, isolating or concentrating the polynucleic acids present in the sample;

(ii) when appropriate, at least one of the sets of primers as defined above;

(iii) at least one of the probes as defined above, possibly fixed to a solid support;

(iv) a hybridization buffer, or components necessary for producing said buffer;

(v) a wash solution, or components necessary for producing said solution;

(vi) when appropriate, a means for detecting the hybrids resulting from the preceding hybridization;

(vii) when appropriate, a means for attaching said probe to a solid support

The following examples only serve to illustrate the present invention. These examples are in no way intended to limit the scope of the present invention.

FIGURE AND TABLE LEGENDS

Figure 1: Natural and drug induced variability in the vicinity of codons 41, 50, 67-70, 74-

75, 150, 181-184, 215 and 219 of the HIV RT gene. The most frequently observed wild-type sequence is shown in the top line. Naturally occuring variations are indicated below. Drug-induced variants are indicated in bold italics

Figure 2 A . Reactivities of the selected probes for codon 41 immobilized on LiPA strips with reference material. The position of each probe on the membrane strip is shown at the right of each panel. The sequence of the relevant part of the selected probes is given in Table 4. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are indicated in Table 4. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. *: False positive reactivities. On top of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

Figure 2 B. Reactivities of the selected probes for codons 69-70 immobilized on LiPA strips with reference material. The position of each probe on the membrane strip is shown at the right of each panel. The sequence of the relevant part of the selected probes is given in Table 4. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are indicated in Table 4. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. *: False positive reactivities. On top of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

Figure 2 C. Reactivities of the selected probes for codons 74-75 immobilized on LiPA strips with reference material. The position of each probe on the membrane strip is shown at the right of each panel. The sequence of the relevant part of the selected probes is given in Table 4. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are indicated in Table 4. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. On top of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

Figure 2 D. Reactivities of the selected probes for codon 184 immobilized on LiPA strips

with reference material. The position of each probe on the membrane strip is shown at the right of each panel. The sequence of the relevant part of the selected probes is given in Table 4. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are indicated in Table 4. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. On top of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

Figure 2 E. Reactivities of the selected probes for codon 215 immobilized on LiPA strips with reference material. The position of each probe on the membrane strip is shown at the right of each panel. The sequence of the relevant part of the selected probes is given in Table 4. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are indicated in Table 4. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. On top of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

Figure 2 F. Reactivities of the selected probes for codon 219 immobilized on LiPA strips with reference material. The position of each probe on the membrane strip is shown at the right of each panel. The sequence of the relevant part of the selected probes is given in Table 4. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are indicated in Table 4. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. On top of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

Figure 3. Clinical and virological features detectable in three patient follow-up samples. All three patients were infected with a HIV-1 strain showing the M41-T69-K70-L74-V75-M184-F214-T215-K219 genotype (wild type pattern). Top: Fluctuations between plasma HIV RNA copy numbers (■) and CD4 cell count (x) are given in function of time. The different treatment regimens and the period of treatment is indicated on top. Middle: Changes that appeared during the treatment period and that could be scored by means of

the LiPA probes are indicated, for patient 91007 at codon 41 and 215; for patient 94013 at codon 184; for patient 92021 at codon 70, 214, 215, 219. Bottem: Corresponding LiPA strips for a subset of the aa changes are shown. LiPA probes are indicated on the left, the aa interpretation is indicated at the right of each panel.

Figure 4. Reactivities of the selected probes for codons 151 and 181 on LiPA strips with reference material. The position of each probe on the membrane strip is shown at the right of each panel. The sequence of the relevant part of the selected probes is given in Table 3. LiPA strips were incubated with sequence-confirmed PCR fragments, extracted and amplified from: a wild-type HIV-1 isolate (strip 1), a wild-type isolate with a polymorphism at codon 151 (strip 2) or 149 (strip 3), a multi-drug resistant HIV-1 isolate (strip 4) with no information about codon 181 and a non-nucleoside analogue treated HIV-1 isolate which remained wild-type at codon 151 (strip5).

Table 1: Mutations in HIV-1 RT gene associated with resistance against nucleoside RT inhibitors. More details are given in Mellors et al., 1995.

Table 2: Mutations in HIV-1 RT gene associated with resistance against HIV-1 specific RT inhibitors. For more details see Mellors et al., 1995.

Abbreviations in Table 1 and 2:

AZT : 3'-azido-2'3'-dideoxythymidine

ddC : 2'3'-dideoxycytidine

ddl : 2'3'-dideoxyinosine

3TC : 3'dideoxy-3'-thiacytidine

FTC : 2'3'-dideoxy-5-fluoro-3'-thiacytidine

L'697,593 : 5-ethyl-6-methyl-3-(2-phthalimido-ethyl)pyridin-2(1H)-one

L'697,661 : 3-Il(-4,7-dichloro-1,3-benzoxazol-2-yl)methyl amino-5-ethyl-6-methylpyridin-2(1H)-one

Nevirapine : 11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyridol(3,2-b:2',3'-e)diazepin-6-one

TIBO R82150 : (+)-(5S)-4,5,6,7,-tetrahydro-5-methyl-6-(3-methyl-2

butenyl)imidazo(4,5,1-)k) (1,4)-benzodiazepin-2(1H)-thione

TIBO 82913 : (+)-(5S)-4,5,6,7,-tetrahydro-9-chloro-5-methyl-6-(3-methyl-2-butenyl)imidazo(4,5,1 kj)-(1,4)benzo-diazepin-2(1H)-thione

TSAO-m^{2}T : (2',5'-bis-o-(tert-buthyldimethylsilyl)-3'-spiro-5'-(4'-amino-1',2'-oxathiole-2',2'-dioxide)

U90152 : 1-(3-(1-methylethyl)-amino)-2-pyridinyl)-4-(5-(methylsulphonyl)-amino)-1H-indol-2yl)-carbonyl)-piperazine

Table 3: HIV RT wild-type and drug resistance mutation probes. The probes witheld after selection are indicated as "y".

Table 4: Prediction and prevalence of LiPA probe reactivity. Probe names corresponding with the selected motifs are presented in the left column, with the relevant part of each probe shown under the consensus. The prevalence of these motives, determined using panels of European and US sera, is given in the middle column. The right column indicates the corresponding strips of Figure 2 A-F and the accession number of the reference panel clone used to obtain this reactivity.

EXAMPLES

Example 1:

a. Materials and methods.

Plasma samples were taken from HIV type-1 infected patients and stored at -20°C until use. Patients were treated with AZT, ddI, ddC, D4T, 3TC, or several combinations of these prodrugs. The European serum samples tested were randomly selected. For the US serum collection, only the first sample from a follow-up series was taken. Some of these US patients were treated, others were not treated.

HIV RNA was prepared using the guanidinium-phenol procedure. Fifty μl plasma was mixed with 150 μl Trizol®LS Reagent (Life Technologies, Gent, Belgium) at room temperature (volume ratio: 1unit sample/ 3 units Trizol). Lysis and denaturation occured by carefully pipetting up and down several times, followed by an incubation step at room temperature for at least 5 minutes. Fourty μl $CHCl_3$ was added and the mixture was

shaken vigorously by hand for at least 15 seconds, and incubated for 15 minutes at room temperature. The samples were centrifuged at maximum 12,000g for 15 minutes at 4°C, and the colourless aquous phase was collected and mixed with 100 μl isopropanol. To visualize the minute amounts of viral RNA, 20 μl of 1μg/μl Dextran T500 (Pharmacia) was added, mixed and left at room temperature for 10 minutes. Following centrifugation at max. 12,000g for 10 minutes at 4°C and aspiration of the supernatant, the RNA pellet was washed with 200 μl ethanol, mixed by vortexing and collected by centrifugation at 7,500g for 5 minutes at 4°C. Finally the RNA pellet was briefly air-dryed and stored at -20°C.

<u>For cDNA synthesis and PCR amplification</u>, the RNA pellet was dissolved in 15 μl random primers (20 ng/μl, pdN_6, Pharmacia), prepared in DEPC-treated or HPLC grade water. After denaturation at 70°C for 10 minutes, 5 μl cDNA mix was added, composed of 4 μl 5x AMV-RT buffer (250mM Tris.HCl pH 8.5, 100mM KCl, 30mM $MgCl_2$, 25 mM DTT), 0.4 μL 25mM dXTPs, 0.2 μl or 25U Ribonuclease Inhibitor (HPRI, Amersham), and 0.3 μl or 8U AMV-RT (Stratagene). cDNA synthesis occured during the 90 minutes incubation at 42°C. The HIV RT gene was than amplified using the following reaction mixture: 5 μl cDNA, 4.5 μl 10x Taq buffer, 0.3 μl 25 mM dXTPs, 1 μl (10 pmol) of each PCR primer, 38 μl H_2O, and 0.2 μl (1 U) Taq. The primers for amplification had the following sequence: outer sense RT-9: 5' bio-GTACAGTATTAGTAGGACCTACACCTGTC 3' (SEQ ID NO 96); nested sense RT-1: 5' bio-CCAAAAGTTAAACAATGGCCATTGACAGA 3' (SEQ ID NO 97); nested antisense RT-4: 5' bio-AGTTCATAACCCATCCAAAG 3' (SEQ ID NO 98); and outer antisense primer RT-12: 5' bio-ATCAGGATGGAGTTCATAACCCATCCA 3' (SEQ ID NO 99). Annealing occured at 57°C, extension at 72°C and denaturation at 94°C. Each step of the cycle took 1 minute, the outer PCR contained 40 cycles, the nested round 35. Nested round PCR products were analysed on agarose gel and only clearly visible amplification products were used in the LiPA procedure. Quantification of viral RNA was obtained with the HIV Monitor™test (Roche, Brussels, Belgium).

<u>Selected PCR products</u>, amplified without 5' biotine primers, were cloned into the pretreated EcoRV site of the pGEMT vector (Promega). Recombinant clones were selected after α-complementation and restriction fragment length analysis, and sequenced with plasmid primers and internal HIV RT primers. Other biotinylated fragments were directly sequenced with a dye-terminator protocol (Applied Biosystems) using the amplification

primers. Alternatively, nested PCR was carried out with analogs of the RT-4 and RT-1 primers, in which the biotine group was replaced with the T7- and SP6-primer sequence, respectively. These amplicons were than sequenced with an SP6- and T7-dye-primer procedure. Sequence information was submitted to the GENBANK.

Probes were designed to cover the different polymorphisms and drug induced mutations. In principle, only probes that discriminated between one single nucleotide variation were retained. However, for certain polymorphisms at the extreme ends of the the probe, cross-reactivity was tolerated. Specificity was reached for each probe individually after considering the % (G+C), the probe lenght, the final concentration of the buffer components, and hybridization temperature. Optimized probes were provided enzymatically with a poly-T-tail using the TdT (Pharmacia) in a standard reaction condition, and purified via precipitation. Probe pellets were disolved in standard saline citrate (SSC) buffer and applied as horizontal parallel lines on a membrane strip. Control lines for amplification (probe 5' CCACAGGGATGGAAAG 3', HIV RT aa 150 to aa 155) and conjugate incubation (biotinylated DNA) were applied alongside. After fixation of the probes onto the membranes by baking, membranes were sliced into 4mm strips.

To perform LiPA tests, equal amounts (10 μl) of biotinylated amplification products and denaturation mixture (0.4 N NaOH/0.1% SDS) were mixed, followed by an incubation at room temperature for 5 minutes. Following this denaturation step, 2 ml hybridization buffer (2xSSC, 0.1% SDS, 50mM Tris pH7.5) was added together with a membrane strip and hybridization was carried out at 39°C for 30 min. Then, the hybridization mixture was replaced by stringent washing buffer (same composition as hybridisation buffer), and stringent washing occured first at room temperature for 5 minutes and than at 39°C for another 25 minutes. Buffers were than replaced to be suitable for the streptavidine alkaline phosphatase conjugate incubations. After 30 minutes incubation at room temperature, conjugate was rinsed away and replaced by the substrate components for alkaline phosphatase, Nitro-Blue-Tetrazolium and 5-Bromo-4-Chloro-3-Indolyl Phosphate. After 30 minutes incubation at room temperature, probes where hybridization occured became visible because of the purple brown precipitate at these positions.

b. Results.

b.1 The HIV-1 RT gene PCR and selection of a reference panel.

PCR primers were chosen outside the target regions for probe design. The amplified region located inside the nested primers covered the HIV-1 RT gene from codon 29 to codon 220. The primer design was based on published sequences from the HIV-1 genotype B clade. European and United States HIV-1 positive serum samples, stored appropriately (at -20°C) without repeating freezing-thawing cycles, were PCR positive in 96% of the cases (not shown). The annealing temperature for the selected primers seemed to be crucial (57°C). At 55°C, a second aspecific amplicon of approximately 1500-bp was generated; and at 59°C the amount of specific fragment decreased drastically. With the current primer combination, the corresponding RT region could be amplified from isolates of the genotype A, C, D and F clade, but with a reduced sensitivity.

A total of 25 selected PCR fragments with the target polymorphisms and mutations were retained as reference panel and sequenced on both strands. The selection occurred during the evaluation of the probes, and these samples originated from naive or drug-treated European or US patients. Biotinylated PCR products from this panel (Accession Number L78133 to L78157) were used to test probes for specificity and sensitivity.

b.2 Nucleotide target region for probe design and probe selection.

Table 4 and parts of Figure 1 are a compilation of the natural and drug-selected variability in the vicinity of aa 41, 69-70, 74-75, 184, 215, and 219 of the HIV RT gene. To create this table and parts of this figure, the "National Centre for Biotechnology Information" database was searched and all HIV-1 genome entries were retrieved and analyzed one by one. Only those entries displaying non-ambiguous sequence information in the vicinity of the above-mentioned codons were retained for further interpretation. It should be noted that the indicated variations do not imply that they occur in the same sequence: for example the variability observed at codon 40 and 43 may occasionally occur together, but most often, if they occur, only one of them is found. In these 6 regions, a total of 19 different third-letter and two first-letter (codon 43 AAG versus GAG and codon 214 TTT versus CTT) polymorphisms need to be included in the selection of wild type probes. Another 13 first-letter and/or second-letter variations are drug-induced and are the main targets for the selection of probes (Figure 1).

For the design of relevant probes, only those database motifs that systematically returned (highly prevalent motif) were included, while scattered mutations which were found randomly (low prevalent motif, not shown) were ignored. Based on database

sequences, seven motifs for codon 41 (91.6% of all entrees), 6 for codon 69-70 (86.2%), 2 for codon 74-75 (90.4%), 5 for codon 184 (96.6%), 9 for codon 215 (94.1%), and 2 for codon 219 (88.2%) were selected (Table 4).

Probe names corresponding with the selected motifs are presented in the left column of Table 4, with the relevant sequence part of each probe shown under the consensus. The prevalence of these motives was than determined using panels of European and US sera (Table 4).

In many cases, the database entries were not representative for the samples tested. Upon analyzing the European and US samples, many were not reactive with these database- selected probes. Upon sequencing analysis of several of these unreactive PCR products, another 8 motifs became apparent, for which the corresponding probes were designed (41w20, 41m12, 70m13, 74w9, 74m6, 74m12, 184w24, 215m49). By including these newly designed motifs, negative results were markedly decreased; for all codon positions except codon 219, the total percentage of reactivity exceeds 90%.

Another 4 probes were designed (41m11, 215m50, 219m7, and 219m9) because their sequence motif was found in the cloned reference panel, although no reactivity with the tested plasma virus samples was found so far. The existence of these rare sequence motifs is explained by assuming that they exist at an extremely low frequency in the viral quasispecies, remaining undetectable by direct detection methods, but becoming apparent after cloning.

The sequence motif of probe 215m13 was generated in recombinant clones by site-directed mutagenesis (not shown). The rational behind this probe design was to determine whether the sequence combination of codon Y215 (TAC) can occur in combination with L214 (CTT) *in vivo*. However, this latter motif was not found in the plasma samples tested.

Four probes (41w15, 70w8, 215w29, 215w27) are in fact redundant, because they detect identical sequence motifs covered by other probes. However, the location of these redundant probes is slightly different to their sequence-identical counterpart. These probes have the potential to avoid negative results which might otherwise appear as a consequence of random mutations in the probe target area and can therfore increase the specificity of recognition.

b.3 Probe specificity and sensitivity.

The 48 selected probes were applied separately on LiPA strips. Biotinylated PCR fragments generated from the reference panel or directly from plasma virus were alkali-denatured, the hybridization buffer and LiPA strips were added, and submitted to stringent hybridization and washing conditions. Positions where hybridization occurred were revealed by the biotine-streptavidine colorimetric detection system. Figure 2 (A to F) shows the reactivity of these 48 designed probes. In the right columns of Table 4, there is the indication of the corresponding strip in Figure 2, and the accession number of the reference panel clone used to obtain this reactivity. The reactivities of these probes were concordant with the nucleotide sequences. False positive reactivities were observed only for probe 41w19 (Fig. 2A.9) and for 70m3 (Fig. 2B.8), with extremely rare sequence motifs 41m12 (prevalence less than 0.3%) and 70m16 (not experimentally found), respectively. Weak cross-reactivity, as was observed on probe 41m13 with a 41m27 motif (Figure 2A.10) was, in general, not tolerated in the probe design. When occurring, however, it never influenced the genotypic resistance interpretation.

b.4 Applicability of the LiPA in patient management.

We selected follow-up samples from three patients and analyzed the viral genotype on the 48 LiPA probes. Figure 3 illustrates the applicability of genotypic resistance measurement in conjunction with the analysis of viral load and CD4 cell count. All three patients had a wild type virus (i.e. M41-T69-K70-L74-V75-M184-F214-T219-K219) strain in the sample collected before anti-retroviral treatment. Codon positions that changed upon treatment are presented in Figure 3.

From Patient 91007, 11 serum samples were analyzed, the first sample being collected 2 weeks before the start of therapy. The LiPA revealed that before treatment, in a T215 context, two variants at codon position 213 were predominantly present (GGG and GGA respectively detected by probe 215w11 and 215w9/215w29). From week 50 until week 81, a mixture of T215 and Y215 could be detected. Both variants at codon 213 were also represented in the selected resistant genotypes (probes 215m17 and 215m14 are positive). From week 94 onwards, only Y215 mutant virus could be detected. A nearly identical geno-conversion at codon 41 was observed, with the detection of mixtures (M41 and L41) from week 81 until week 111; from week 126 onwards, only L41 could be found (strips not shown). CD4 values were highly variable. Nevertheless, a continuous decrease in CD4 is apparent (p=0.019, linear regression analysis). Viral load also decreased

initially. However, the direct response to the treatment might have been missed in this follow-up series, since the first sample after the start of the treatment is at 32 weeks. From than on, viral load increased.

Patient 94013 was treated with 3TC monotherapy from week 2 onwards. At week 10, a mixture of M184 and V184 could be detected. From week 14 on, only V184 was present. CD4 counts increased nearly 2.5-fold, with the highest level at week 10. Viral load decreased spectacularly by 3 log units. But from week 10 onwards, a slight but steady increase to week 23 was noted. The decrease in CD4 and increase in viral load coincided with the appearance of the V184 motif.

Patient 92021 was followed for 55 weeks. AZT treatment started at week 10, followed by a supplemental ddC treatment from week 20 onwards. The first sample was found to be reactive with probe 215w9/w29 (F214T215 = TTTACC), but trace amounts of reactivity with 215w53 (L214T215 = TTAACC) could be detected as well, indicating the presence of at least two variants at that time. From week 19 onwards, the codon L214 = TTA motif became more important. At week 42, the first sign of genotypic resistance could be detected by the presence of a F214Y215 motif (TTTTAT). Finally at week 55, only F214Y215 could be detected. The L214 = TTA motif disappeared completely. At week 42, a mixture (K and R) at codon 70 was present, but at week 55, only R70 could be detected. At week 55, a mixture of codon 219 motifs (K and E) was found (strips not shown). CD4 initially increased, with a maximal effect during AZT monotherapy peaking at week 21. From then on, a continuous decrease was observed. However, ten weeks of AZT treament did not result in a drop in viral load, since the values of week 16 and 19 were nearly unchanged. It is only after start of the combination therapy (week 20) that the viral load dropped by 1.67 log. From this patient, it is tempting to assume that L214T215 confers genotypic resistance to AZT treatment, and that the addition of ddC is necessary to induce the natural F214Y215 genotype. The rise in CD4 cell count may be the consequence of the drug itself, and not from drug-induced protection (Levy et al. 1996).

c. Discussion

By adapting the previously designed LiPA technology (Stuyver et al. 1993) for the HIV RT gene, the described assay format permits the rapid and simultaneous detection of wild type and drug-selected variants associated with the genotypic resistance for AZT, ddI, ddC, d4T, FTC and 3TC. The Inno LiPA HIV drug-resistance strip provides information

about the genetic constitution of the RT gene in the vicinity of codon 41, 69, 70, 74, 75, 184, 215, and 219 at the nucleotide and, hence, also at the deduced protein level. Essentially, the biotinylated RT PCR product is hybridized against immobilized specific oligonucleotides (Table 4), which are directed against the indicated codon variabilities. Following this reverse-hybridization, the oligonucleotide-biotinylated-PCR-strand hybrid is recognized by the streptavidine-alkaline phosphate conjugate, which then in turn converts the alkaline phosphate substrate into a purple brown precipitate.

Using this assay, we studied the specificity and reactivity of 48 probes, covering 6 different regions. This combination should allow the reliable detection of most of the genetic resistance-related codon combinations observed to date. Occasionally occurring mutations in the vicinity of the target codons, not taken into consideration during probe design, may eventually prevent hybridization of the probes for a particular target region. This problem is partially solved by the redundancy of probes at the most important codons. Results obtained using 358 HIV infected plasma samples showed that, depending on the codon position under investigation, between 82.4% and 100% of the combinations could be detected, or an average of 92.7%. It is important to mention here that the assay was developed for resistence detection of the HIV-1 genotype B, and only limited information is currently available about the outcome of this assay with other genotypes. Since the amplification primer combination is more or less universal for all the HIV-1 isolates, some of the indeterminate results may well be due to the presence of non-genotype B virus strains.

So far, several assays for the detection of the wild-type and drug-selected mutations in the HIV RT gene have been described. These include Southern blotting (Richman et al., 1991), primer-specific PCR (Larder et al., 1991), PCR-LDR (Frenkel et al., 1995), RNAse A mismatch cleaving (Galandez-Lopez et al., 1991), and hybridization against enzyme-labeled probes (Eastman et al., 1995). The general advantage of the LiPA and other genotypic assays is the speed by which results are obtained when compared to phenotypic assays. The particular advantage of our test is its multi-parameter (in this particular case multi-codon) format. Moreover, the assay can easily be extended not only for the screening of the other RT-codons, but also for proteinase codons associated with resistance (Mellors et al., 1995). As was illustrated in Figure 3, mixtures of wild-type and drug-selected mutations can be detected easily. The detection limit for these mixtures is

dependent on the sensitivity of the probes, but reliable results can be obtained as soon as 5 to 10% of the minor component is present (not shown). We were unable to provide reliable evidence for mixtures with any sequencing protocol at the same sensitivity level.

Due to the large amount of variables that need to be included in the selection of specific probes (temperature of hybridization, ionic strength of hybridization buffer, length of the probe, G+C content, strand polarity), it might occasionally occur that some of the probes will show weak false positive reaction with related but hitherto unreported sequences. In our experience, and if this occurred, this has never influenced the interpretation at the deduced aa level. In the current selection of probes, all except two (41w19 and 70m3) were retained on the basis of 100% specificity: as soon as one nucleotide differs in the probe area, hybridization is abolished. Further fine-tuning of these two probes will therefore be necessary to obtain the required specificity.

Accompanying polymorphisms in the vicinity of the target codons are found with a rather high prevalence in wild-type virus strains, but not in mutant sequences. A partial list of such combinations is hereby presented: codon V74=GTA without polymorphism at codon 73, 75 or 76; codon V184=GTG without codon Q182=CAG; and codon F215=TTC without F214=TTC/TTA or L214=CTT. The most intriguing example is the following: L214T215 (CTTACC) is predicted for approximately 7.8% of the wild type sequences. The corresponding motif L214Y215 (CTTTAT) apparently does not exist in plasma virus. From the example shown in Figure 3, it is clear that selection of mutants is a very flexible and complex phenomena. In this particular case, viruses having codon F214 were replaced by a L214 viral population in the AZT monotherapy period, but upon selecting for genotypic drug resistance at codon 215, the original F214 configuration was restored. Clearly, the selection for the Y215 genotype prohibits the presence of a L214 genotype. Since no evidence has yet emerged that L214 confers resistance to anti-retroviral compounds, the appearance of this special mutant during the AZT monotherapy period is difficult to interpret. More research will certainly be necessary to clarify this issue. But if L214 should indeed provide low-level genotypic resistance to AZT treatment, approximately 7.8% of the naive infections will not benefit from initial AZT monotherapy.

Since antiviral treatment can result in a marked extension of life expectancy for HIV infected patients, it is of utmost importance to find the best drug regimen for each individual separately. Therefore, monitoring of the magnitude and duration of the virus

load and CD4 cell changes is a prerequisite. However, knowledge concerning the genetic constitution of the virus may also be an important factor in designing optimal treatment schedules. Optimizing therapies making good use of available information (viral load, CD4 cell count, genetic resistance) has remained largely unexploited. If this was partially due to the complexity of screening for all the mutational events, the above-described LiPA technology should remove one key obstacle.

In conclusion, we have described a genotypic assay for the detection of wild type and drug selected codons in the HIV RT gene. The combination of the assay result along with viral load and CD4 cell monitoring should permit better design of patient-dependent optimal treatment schedules.

Example 2:

Multi-Drug Resistant (MDR) HIV-1 isolates have been described. These MDR isolates are characterized by having mutations in their genome, compared to the wild type HIV-1 genome, which result in a set of amino acid changes. A key mutation leading to multi-drug resistance was found to be localized in codon 151 of the HIV-1 RT gene. Consequently, and as detecting these MDR isolates is clinically important, we designed probes recognizing wild-type (probe 151w2) and mutant (probes 151m4 and 151m19) HIV-1 isolates. Furthermore, the presence of polymorphisms in the direct vicinity of codon 151 (codon 149) and at codon 151 have been described. Therefore, we also designed two additional probes (probes 151w6 and 151w11) which detect these polymorphisms (Figure 4 and Table 3).

Treatment with non-nucleoside analogues, such as Nevirapine (Boehringer Ingelheim), selects for several amino acid changes in conserved regions of the HIV-1 RT gene. One of the most important amino acid changes is Y181C, a codon change that confers high level resistance. As the detection of this mutation is also clinically important, we designed probes recognizing the wild-type (181w3 and 181w5) and mutant (181m7) isolates (Figure 4 and Table 3).

Figure 4 shows the application of the selected probes for codon 151 and 181. The position of the probes on the strips is indicated on the right side of the strips. LiPA strips were incubated with sequence-confirmed PCR fragments, extracted and amplified from: a wild type HIV-1 isolate (strip 1), a wild type HIV-1 isolate with a polymorphism at codon

151 (strip 2) or codon 149 (strip 3), a multi-drug resistant HIV-1 isolate (strip 4) with no information about codon 181 and a non-nucleoside analogue-treated HIV-1 isolate which remained wild type at codon 151(strip 5).

Table 1

AZT	M41L	ATG to TTG or CTG
	D67N	GAC to AAC
	K70R	AAA to AGA
	T215Y	ACC to TAC
	T215F	ACC to TTC
	K219Q	AAA to CAA
	K219E	AAA to GAA
ddl	K65R	AAA to AGA
	L74V	TTA to GTA
	V75T	GTA to ACA
	M184V	ATG to GTG
ddC	K65R	AAA to AGA
	T69D	ACT to GAT
	L74V	TTA to GTA
	V75T	GTA to ACA
	M184V	ATG to GTG
	Y215C	TTC to TGC
d4T	I50T	ATT to ACT
	V75T	GTA to ACA
3TC or FTC	M184V	ATG to GTG or GTA
	M184I	ATG to ATA
1592U89	K65R	AAA to AGA
	L74V	TTA to GTA
	Y115F	TAT to TTT
	M184V	ATG to GTG

Table 2

Nevirapine	A98G	GCA to GGA
	L100I	TTA to ATA
	K103N	AAA to AAC
	V106A	GTA to GCA
	V108I	GTA to ATA
	Y181C	TAT to TGT
	Y181I	TGT to ATT
	Y188C	TAT to TGT
	G190A	GGA to GCA
TIBO	L199I	TTA to ATA
R82150		
TIBO	L100I	TTA to ATA
R82913	K103N	AAA to AAC
	V106A	GTA to GCA
	E138K	GAG to AAG
	Y181C	TAT to TGT
	Y188H	TAT to CAT
	Y188L	TAT to TTA
L697.593	K103N	AAA to AAC
	Y181C	TAT to TGT
L697,661	A98G	GCA to GGA
	L100I	TTA to ATA
L697,661	K101E	AAA to GAA
	K103N	AAA to AAC
	K103Q	AAA to CAA
	V108I	GTA to GCA
	V179D	GTT to GAT
	V179E	GTT to GAG
	Y181C	TAT to TGT
BHAP		

U-90152	P236L	CCT to CTT
BHAP	K101E	AAA to GAA
U-87201	K103N	AAA to AAC
	Y181C	TAT to TGT
	Y188H	TAT to CAT
	E233V	GAA to GTA
	P236L	CCT to CTT
	K238T	AAA to ACA
BHAP	L100I	TTA to ATA
U-88204	V106A	GTA to GCA
	Y181C	TAT to TGT
	Y181I	TGT to ATT
HEPT	Y188C	TAT to TGT
E-EBU	Y181C	TAT to TGT
E-EBU-dM	Y106A	GTA to GCA
E-EPU and	Y181C	TAT to TGT
E-EPSeU	Y188C	TAT to TGT
a-APA	Y181C	TAT to TGT
R18893		
S-2720	G190E	GGA to GAA
TSAO	E138K	GAG to AAG
BM+51.0836	Y181C	TAT to TGT

Table 3

HIV RT wild-type and drug resistance

Formula	probe	Sequentie oligo	SEQ ID NO	PROBE selection
wild-type probes for position M41				
E40M41K43	41w7	AGAAATGGAAAAGGA	1	y
E40M41K43	41w15	TGTACAGAAATGGAA	2	y
M41K43	41w16	AAATGGAAAAGGAAG	3	
E40M41	41w18	TACAGAGATGGAAA	4	
E40M41K43	41w19	GTACAGAGATGGAAA	5	
E40M41K43	41w20	AGAGATGGAAAAAGA	6	y
E40M41K43	41w30	AGAAATGGAGAAGGA	7	y
E40M41	41w31	ACAGAGATGGAAAA	8	
E40M41	41w32	GTACAGAGATGGAA	9	y
E40M41K43	41w33	CAGAGATGGAAAAG	10	
E40M41K43	41w34	AGAAATGGAAAAAGA	11	
E40M41K43	41w35	GAAATGGAAAAAGA	12	
E40M41K43	41w36	CAGAAATGGAAAAAGA	13	y
E40M41K43	41w37	AGAAATGGAAAAAGAA	14	
drug-induced variant probes for position L41				
E40L41K43	41m8	AGAATTGGAAAAGGA	15	
E40L41K43	41m11	AGAGTTGGAAAAGGA	16	y
E40L41K43	41m12	AGAGCTGGAAAAGG	17	y
E40L41K43	41m13	AGAACTGGAAAAGG	18	y
E40L41K43	41m14	GAGCTGGAAAAGG	19	
E40L41K43	41m21	ACAGAATTGGAAAAG	20	y
E40L41	41m22	ACAGAATTGGAAAA	21	
E40L41	41m23	ACAGAACTGGAAAA	22	
E40L41K43	41m24	AGAATTGGAAGAGG	23	y
E40L41E43	41m25	CAGAATTGGAAGAGG	24	
E40L41E43	41m26	AGAATTGGAAGAGGA	25	
E40L41E43	41m27	AGAACTGGAAGAGG	26	y
E40L41E43	41m28	CAGAACTGGAAGAGG	27	
E40L41E43	41m29	AGAACTGGAAGAGGA	28	
wild-type probes for positions I50 or V50 or T50				
K49I50	50w4	CAAAAATTGGGCCT	29	y
R49I50	50w9	ATTTCAAGAATTGGG	30	y
K49V50	50w5	TTCAAAAGTTGGGC	31	y
K49I50	50w13	CAAAAATCGGGCCTG	32	y
K49T50	50w14	AAAAATCGGGCCTGA	33	y
wild-type probe for position D67				
K64K65K66D67	67w4	AAAGAAGAAAGACAG	34	y
drug-induced variant probe for position N67				
K64K65K66N67	67m19	ATAAAGAAAAAGAACAGTA	35	y
wild-type probes for positions T69 or K70				
T69K70	70w1	AGTACTAAATGGAGAA	36	y
D69K70	70w2	AGTGATAAATGGAGAA	37	y
T69K70	70w8	ACAGTACTAAATGGAG	38	y
K70K73	70w11	TAAATGGAGAAAAITAG	40	
drug-induced variant probes for positions D69 or N69 or A69 or R70				
D69R70	70m3	GTGATAGATGGAGAA	41	

T69R70	70m6	GTACTAGATGGAGA	42	
T69R70	70m12	AGTACTAGATGGAGA	43	y
T69R70	70m13	AGTACAAGATGGAGA	44	y
N69R70	70m14	CAGTAATAGATGGAG	45	y
A69R70	70m15	ACAGTGCTAGATGGA	46	
A69R70	70m16	CAGTGCTAGATGGA	47	y
A69R70	70m17	CAGTGCTAGATGGA	48	
D69R70	70m18	CAGTGATAGATGGA	49	y
D69R70	70m19	CAGTGATAGATGGAG	50	
D69R70	70m20	AGTGATAGATGGAG	51	
D69R70	70m21	AGTGATAGATGGAGA	52	
wild-type probes for positions L74 or V75				
K73L74V75D76	74w5	GAGAAAATTAGTAGATTT	53	y
K73L74V75D76	74w8	AAAATTAGTAGACTTC	54	y
K73L74V75D76	74w9	GAGAAAGTTAGTGGATT	55	
drug-induced variant probes for positions V74 or T75				
K73V74V75D76	74m6	AGAAAAGTAGTAGATTT	56	y
K73L74T75D76	74m10	AAAATTAACAGATTTC	57	
K73L74T75D76	74m11	GAAAATTAACAGATTT	58	
K73L74T75D76	74m12	GAAAATTAACAGATTTC	59	y
wild-type probes for position Q151				
P150Q151G152	151w2	CTTCCACAGGGATGG	60	y
P150Q151G152	151w6	CTTCCACAAGGATGG	61	y
P150Q151G152	151w11	TGCTCCCACAGGGATG	62	y
drug-induced variant probe for position M151				
P150M151G152	151m4	CTTCCAATGGGATGG	63	y
P150M151G152	151m19	GCTTCCAATGGGATGG	64	y
wild-type probe for position Y181				
Y181	181w3	AGTTATCTATCAATACAG	65	y
drug-induced variant probe for position C181				
C181	181m7	AGTTATCTGTCAATAC	66	y
wild-type probes for position M184				
Q182M184	184w11	TCAATACATGGATGAGG	67	y
Q182M184	184w17	TCAGTACATGGATGAGG	68	y
Q182M184	184w18	ATCAATACATGGATGA	69	
Q182M184	184w19	TCAGTACATGGATG	70	
Q182M184	184w21	ATCAATATATGGATG	71	y
Q182M184	184w22	ATCAATATATGGATGA	72	
Q182M184	184w23	TCAATATATGGATGA	73	
Q182M184	184w24	TCAATACATGGACGA	74	y
Q182M184	184w25	CAATACATGGACGAT	75	
Q182M184	184w26	TCAATACATGGACGAT	76	
drug-induced variant probes for position V184 or I184				
Q182V184	184m12	CAATACGTGGATGAGGG	77	y
I184	184m13	AATACATAGATGAT	78	
Q182I184	184m14	CAATACATAGATGAT	79	
Q182I184	184m15	CAATACATAGATGATT	80	
Q182V184	184m16	CAATACGTAGATGAT	81	
Q182V184	184m20	TCAATACGTGGATGA	82	
Q182I184	184m27	TCAATACATAGATGAT	83	
Q182I184	184m28	ATCAATACATAGATGAT	84	y

wild-type probes for position T215

G213F214T215	215w9	GGATTTACCACACCA	85	y
L214T215	215w10	GACTTACCACACCA	86	y
F214T215	215w11	GGTTTACCACACCA	87	y
F214T215	215w16	GATTTACCACACCA	88	
T215	215w22	TTACTACACCAGAC	89	y
T215	215w24	TTACCACACCAGA	90	
G213L214T215	215w27	TGGGGACTTACCAC	91	y
G213F214T215	215w29	TGGGGATTTACCAC	92	y
G213F214T215	215w32	GGGGTTCACCACAC	93	
G213F214T215	215w33	GGGATTCACCACAC	94	y
G213F214T215	215w34	GGGATTTACCACACCAG	95	
G213L214T215	215w35	TGGGGACTTACCACACC	96	
G213F214T215	215w36	TGGGGGTTTACCACACC	97	
G213F214T215	215w37	GGGATTTACTACACCAG	98	
G213L214T215	215w52	GGGATTAACCACAC	99	
G213L214T215	215w53	GGGGATTAACCACA	100	y
G213L214T215	215w54	TGGGGATTAACCACA	101	
G213L214T215	215w55	GGGGGTTAACCACA	102	
G213L214T215	215w56	GGGGTTAACCACAC	103	
G213L214T215	215w57	TGGGGGTTAACCAC	104	
G213L214T215	215w65	GGGATTGACCACAC	105	
G213L214T215	215w66	GGATTGACCACACC	106	
G213L214T215	215w67	GGGATTGACCACA	107	y
G213L214T215	215w68	GGGACTGACCACA	108	y
G213L214T215	215w69	GGGACTGACCACAC	109	
G213L214T215	215w70	TGGGGGTTAACCACA	110	
G213L214T215	215w71	TGTGGTTAACCCCCA	111	y
G213L214T215	215w51	GGGGCTTACCACAC	112	

drug-induced variant probes for position Y215 or F215

G213L214Y215	215m13	GGACTTTACACACC	113	y
G213F214Y215	215m14	GGGTTTTACACACC	114	y
G213F214F215	215m15	GGATTTTTCACACCA	115	
G213F214Y215	215m17	GGATTTTACACACC	116	y
G213F214Y215	215m38	GGGATTTTACACACCAG	117	
G213F214F215	215m39	GGGATTTTTCACACCAG	118	
G213F214Y215	215m40	GGGATTTTACACAC	119	
G213F214Y215	215m41	GGGGATTTTACACA	120	
G213F214Y215	215m43	CCCTAAAATGTGTG	121	
G213F214F215	215m44	GGATTTTTCACACC	122	
F214F215	215m45	GATTTTTCACACCA	123	y
G213F214F215	215m46	GGGATTTTTCACAC	124	
G213F214Y215	215m42	CCCCTAAAATGTGT	125	
F214Y215	215m47	GGTTTTATACACCA	126	
G213F214Y215	215m48	GGGTTTTATACACC	127	
G213F214Y215	215m49	GGGGTTTTATACAC	128	y
G213L214T215	215m50	GGGGGCTTACCACA	129	y
G213F214Y215	215m61	GGATTCTACACACC	130	y
F214Y215	215m62	GATTCTACACACC	131	
G213F214Y215	215m63	GGATTCTACACAC	132	
G213F214Y215	215m64	GGGATTCTACACAC	133	
G213F214Y215	215m72	GGGTTTTATACCCC	134	
F214Y215	215m73	GGTTTTATACCCC	135	
F214Y215	215m74	GTTTTATACCCCA	136	

wild-type probes for position K219

K219	219w1	ACCAGACAAAAAACA	137	
K219	219w2	ACCAGACAAAAAAC	138	y

BNSDOCID: <WO_____9727332A1_I_>

K219	219w3	CACCAGACAAAAAAC	139	
K219	219w13	CAGACAAGAAACAT	140	
K219	219w14	CCAGACAAGAAACA	141	
K219	219w15	ACCAGACAAGAAACA	142	
K219	219w16	AGACAAAAAGCATC	143	y
K219	219w17	CAGACAAAAAGCAT	144	
K219	219w18	CAGACAAAAAGCATC	145	
K219	219w19	CCAGATAAAAAACA	146	
K219	219w20	ACCAGATAAAAAAC	147	
K219	219w21	CCCAGATAAAAAACA	148	
K219	219w22	CCAGATAAAAAACATC	149	
K219	219w23	CACCAGATAAAAAAC	150	
K219	219w24	CAGACAAGAAACATC	151	
K219	219w25	ACCAGACAAGAAAC	152	

drug-induced variant probes for position Q219 or E219

Q219	219m4	ACCAGACCAAAAACA	153	
E219	219m5	ACCAGACGAAAAACA	154	
Q219	219m6	ACCAGATCAAAAACA	155	
Q219	219m7	ACCAGATCAAAAAC	156	y
Q219	219m8	CACCAGATCAAAAAC	157	
E219	219m9	ACCAGACGAAAAAC	158	y
E219	219m10	CCAGACGAAAAACA	159	
Q219	219m11	CCAGACCAAAAACA	160	
Q219	219m12	ACCAGACCAAAAAC	161	

Table 4: Prediction and prevalence of LiPA probe reactivity

probe	consensus nucleic acid	consensus amino acid	prevalence database	prevalence Europe	prevalence US	prevalence Rp	Corresponding Figure strip	Corresponding Figure Acc.Nb
	Codon 38 - 43		n=191/m=25	n=306	n=52	n=25		
	TGTACAGAAATGGAAAAG	CTEMEK						
41w7	-------------	----	122 (62.9%)	237	35	11	1a.1	L78149
41w15*	----------------	-----	118	230	38	9	1a.1	L78149
41w19	-----G------	----	5 (2.6%)	10	2	2	1a.2	L78156
41w20	--G--------A	----	0	6	0	1	1a.3	L78157
41w30	--------G---	----	1 (0.5%)	8	6	1	1a.4	L78154
41m21	------T--------	--L--	18 (9.4%)	37	7	2	1a.5	L78136
41m11	--GT--------	-L--	0	0	0	1	1a.6	L78140
41m24	---T-----G--	-L-E	12 (6.3%)	1	2	1	1a.7	L78144
41m13	---C--------	-L--	14 (7.3%)	21	3	1	1a.8	L78139
41m12	--GC--------	-L--	0	1	0	1	1a.9	L78155
41m27	---C-----G--	-L-E	3 (1.6%)	0	1	1	1a.10	L78137
		total	175 (91.6%)	95.1%	100%	88%		

Table 4 (cont'd 1)

probe	Codon 68 - 72		n=354/m=32		n=306	n=52	n=25		
	AGTACTAAATGGAGA	STKWR							
70w1	---------------	-----	224	(63.3%)	230	39	13	1b.1,2	L78147
70w8*	------------	----	208		210	38	11	1b.2	L78144
70m12	-------G-------	--R--	37	(10.5%)	46	6	4	1b.3	L78148
70m13	-----A-G-------	--R--	0		0	1	2	1b.4	L78133
70w2	---GA----------	-D---	25	(7.1%)	4	4	2	1b.5	L78136
70m3	GA--G-------	DR--	10	(2.8%)	3	1	0	1b.6	pending
70m14	----A--G----	-NR-	7	(2.0%)	4	5	2	1b.7	L78154
70m16	---G---G----	-AR-	2	(0.6%)	0	0	1	1b.8	L78150
		total	305	(86.2%)	91.8%	94.2%	96%		

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Table 4 (cont'd 2)

probe	Codon 72 - 77		n=364/m=20	n=306	n=52	n=25		
	AGAAAATTAGTAGATTTC	RKLVDF						
74w5	------------------	-----	320 (87.9%)	264	48	16	lc.1	L78150
74w8	-------------C---	-----	9 (2.5%)	34	1	2	lc.2	L78147
74w9	-----G-----G---	-----	0	17	3	2	lc.3	L78137
74m6	------G--------	--V--	0	5	0	3	lc.4	L78149
74m12	------AC----	--T--	0	1	1	1	lc.5	L78136
		total	329 (90.4%)	93.5%	98.1%	96%		

Table 4 (cont'd 3)

probe	Codon 182 - 185		n=322/m=12	n=306	n=52	n=25		
	CAATACATGGAT	QYMD						
184w11	------------	----	285 (88.5%)	267	46	18	1d.1	L78147
184w17	--G---------	----	16 (5.0%)	9	4	3	1d.2	L78137
184w21	-----T------	----	6 (1.9%)	4	2	1	1d.3	L78145
184w24	-----------C	----	0	1	0	1	1d.4	L78144
184m12	------G-----	--V-	1 (0.3%)	8	0	1	1d.5	L78142
184m28	--------A---	--I-	3 (0.9%)	0	0	1	1d.6	L78148
		total	311 (96.6%)	93.8%	98.1%	100%		

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Table 4 (cont'd 4)

probe	Codon 212 - 218		n=321/m=36	n=306	n=52	n=25		
	TGGGATTTACCACACCAGAC	WGFTTPD						
		----	9 (2.8%)	15	3	2	1e.1	L78146
215w11	G------------	-----	142 (44.2%)	178	24	3	1e.2	L78141
215w9	----------------	----	142	105	16	3	1e.2	L78141
215w29ᶠ	-------------	----	9 (2.8%)	8	4	1	1e.3	L78154
215w33	-----C------	L---	25 (7.8%)	10	0	2	1e.4	L78150
215w10	C-----------	--L-	25	14	0	2	1e.4	L78150
215w27ᶠ	------C-----	-L--	0	0	0	1	1e.5	L78145
215m50	--GC--------	-L--	1 (0.3%)	1	3	1	1e.6	L78138
215w53	-----A------	----	3 (0.9%)	10	2	1	1e.7	L78134
215w22	--T---------	--Y-	88 (27.4%)	50	12	7	1e.8	L78144
215m17	------TA----	--Y-	24 (7.5%)	24	1	1	1e.9	L78149
215m14	--G---TA----	--Y-	0	2	0	2	1e.10	L78148
215m49	--G---TAT---	-F--	1 (0.3%)	16	0	1	1e.11	L78135
215m45	---TT------	-LY-	0	0	0	2	1e.12	L78155
215m13	---C--TA----	total	302 (94.1%)	92.8%	90.4%	96%		

BNSDOCID: <WO_____9727332A1_I_>

Table 4 (cont'd 5)

probe	Codon 217 - 220		n=204/ m=12	n=34	n=52	n=26		
	CCAGACAAAAAA	PDEK						
219w2	------------	----	179 (87.7%)	26	42	18	1f.1	L78144
219m4	------C-----	--Q-	1 (0.5%)	2	4	2	1f.2	L78135
219m7	-----TC-----	--Q-	0	0	0	1	1f.3	L78133
219m9	------G-----	--E-	0	0	0	1	1f.4	pending
		total	179 (88.2%)	82.4%	82.7%	84.6%		

n= : amount of sequences (database retrieved), or isolates tested
m= : amount of motifs corresponding with the n database sequences
* : redundant probes
RP : reference panel
The total percentage for European and US samples is not the sum of probe reactivities, but a result of the complete interpretations for these codons.

BNSDOCID: <WO_______9727332A1_I_>

REFERENCES

Asseline U, Delarue M, Lancelot G, Toulme F, Thuong N (1984) Nucleic acid-binding molecules with high affinity and base sequence specificity : intercalating agents covalently linked to oligodeoxynucleotides. Proc. Natl. Acad. Sci. USA 81(11):3297-301.

Barany F. Genetic disease detection and DNA amplification using cloned thermostable ligase. Proc Natl Acad Sci USA 1991; 88: 189-193.

Bej A, Mahbubani M, Miller R, Di Cesare J, Haff L, Atlas R. Mutiplex PCR amplification and immobilized capture probes for detection of bacterial pathogens and indicators in water. Mol Cell Probes 1990; 4:353-365.

Boom R., Sol C.J.A., Salimans M.M.M., et al. Rapid and simple method for purification of nucleic acids. J Clin Microbiol 1990; 28: 495-503.

Compton J. Nucleic acid sequence-based amplification. Nature 1991; 350: 91-92.

Duck P. Probe amplifier system based on chimeric cycling oligonucleotides. Biotechniques 1990; 9: 142-147.

Eastman S, Boyer E, Mole L, Kolberg J, Urdea M, Holodniy M. Nonisotypic hybridisation assay for determination of relative amounts of genotypic human immunodeficiency virus type 1 zidovudine resistance. 1995; 33: 2777-2780.

Frenkel L, Wagner L, Atwood S, Cummins T, Dewhurst S. Specific, sensitive and rapid assay for human immunodeficiency virus type 1 pol mutations asssociated with resistance to zidovudine and didanosine. J. Clin. Microbiology 1995; 33: 342-347.

Guatelli J, Whitfield K, Kwoh D, Barringer K, Richman D, Gengeras T. Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication. Proc Natl Acad Sci USA 1990; 87: 1874-1878.

Iversen A. K. N., R.W. Shafer, K. Wehrly, M.A. Winters, J.I. Mullins, B. Chesebro, and T. Merigan. Multidrug-resistant human immunodeficiency virus type 1 strains resulting from combination antiretroviral therapy. J. Virol. 1996; 70:1086-1090.

Kwoh D, Davis G, Whitfield K, Chappelle H, Dimichele L, Gingeras T. Transcription-based amplification system and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format. Proc Natl Acad Sci USA 1989; 86: 1173-1177.

Kwok S, Kellogg D, McKinney N, Spasic D, Goda L, Levenson C, Sinisky J. Effects of primer-template mismatches on the polymerase chain reaction: Human immunodeficiency views type 1 model studies. Nucl. Acids Res. 1990; 18: 999.

Landgren U, Kaiser R, Sanders J, Hood L. A ligase-mediated gene detection technique. Science 1988; 241:1077-1080.

Larder B, Kellam P, Kemp S. Zidovudine resistance predicted by direct detecion of mutations in DNA from HIV-infected lymphocytes. Aids 1991; 5: 137-144.

Lomeli H, Tyagi S, Printchard C, Lisardi P, Kramer F. Quantitative assays based on the use of replicatable hybridization probes. Clin Chem 1989; 35: 1826-1831.

Matsukura M, Shinozuka K, Zon G, Mitsuya H, Reitz M, Cohen J, Broder S (1987) Phosphorothioate analogs of oligodeoxynucleotides : inhibitors of replication and cytopathic effects of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA 84(21):7706-10.

Mellors J, Larder B, Schinazzi R. Mutations in HIV-1 reverse transcripatse and protease associated with drug resistance. International Antiviral News (1195); 3: 8-13.

Miller P, Yano J, Yano E, Carroll C, Jayaram K, Ts'o P (1979) Nonionic nucleic acid analogues. Synthesis and characterization of dideoxyribonucleoside

methylphosphonates. Biochemistry 18(23):5134-43.

Nielsen P, Egholm M, Berg R, Buchardt O (1991) Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science 254(5037):1497-500.

Nielsen P, Egholm M, Berg R, Buchardt O (1993) Sequence specific inhibition of DNA restriction enzyme cleavage by PNA. Nucleic-Acids-Res. 21(2):197-200.

Saiki R, Walsh P, Levenson C, Erlich H. Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes Proc Natl Acad Sci USA 1989; 86:6230-6234.

Schinazi R, Larder B, Mellors J. Mutations in HIV-1 reverse transcriptase and protease associated with drug resistance. International Antiviral News, 2:72-74.

Stuyver L, Rossau R, Wyseur A, et al. Typing of hepatitis C virus isolates and characterization of new subtypes using a line probe assay. J. Gen. Virol. 1993; 74 : 1093-1102.

Wu D, Wallace B. The ligation amplification reaction (LAR) - amplification of specific DNA sequences using sequential rounds of template-dependent ligation. Genomics 1989; 4:560-569.

CLAIMS

1. Method for determining the susceptibility to antiviral drugs of viruses which contain reverse transcriptase genes and are present in a biological sample, comprising:

(i) if need be releasing, isolating or concentrating the polynucleic acids present in the sample;

(ii) if need be amplifying the relevant part of the reverse transcriptase genes present in said sample with at least one suitable primer pair;

(iii) hybridizing the polynucleic acids of step (i) or (ii) with at least two RT gene probes, with said probes being applied to known locations on a solid support and with said probes being capable of simultaneously hybridizing to their respective target regions under appropiate hybridization and wash conditions allowing the detection of homologous targets, or with said probes hybridizing specifically with a sequence complementary to any of said target sequences, or a sequence wherein T of said target sequence is replaced by U;

(iv) detecting the hybrids formed in step (iii);

(v) inferring the nucleotide sequence at the codons of intrest as represented in any of Figure 1, or Tables 1, 2 or 4 and/or the amino acids of the codons of intrest and/or antiviral drug resistance spectrum, and possibly the type of viral isolates involved from the differential hybridization signal(s) obtained in step (iv).

2. Method according to claim 1, wherein said viruses are HIV strains.

3. Method according to claim 2, wherein said RT gene probes hybridize specifically to one or more target sequences as represented in any of figure 1 or tables 1, 2 or 4.

4. Method according to claim 1, wherein step (iii) consists of hybridizing with at least two probes hybridizing specifically to one or more target codons within region I as represented in Figure 1.

5. Method according to claim 1, wherein step (iii) consists of hybridizing with at least two probes hybridizing specifically to one or more target codon within region II as

represented in Figure 1.

6. Method according to claim 1, wherein step (iii) consists of hybridizing with at least two probes hybridizing specifically to one or more target codons within region III as represented in Figure 1.

7. Method according to claim 1, wherein step (iii) consists of hybridizing with at least two probes hybridizing specifically to one or more target codons within region IV as represented in Figure 1.

8. Method according to claim 1, wherein step (iii) consists of hybridizing with at least two probes hybridizing specifically to one or more target codons within region V as represented in Figure 1.

9. Method according to claim 1, wherein step (iii) consists of hybridizing with at least two probes hybridizing specifically to one or more target codons within region VI as represented in Figure 1.

10. Method according to claim 1, wherein step (iii) consists of hybridizing with at least two probes hybridizing specifically to one or more target codons within region VII as represented in Figure 1.

11. Method according to claim 1, wherein step (iii) consists of hybridizing with at least two probes hybridizing specifically to one or more target codons within region VIII as represented in Figure 1.

12. Method according to claim 1, wherein step (iii) consists of hybridizing with at least one first probe hybridizing specifically to one or more target codons within any region I to VIII as represented in Figure 1 and at least one other second probe hybridizing specifically to one or more target codons within any region I to VIII as represented in Figure 1.

13. Probe on a solid support which is suitable for hybridizing in a method as defined in

any of claims 1 to 12 and which is preferably represented in Table 3.

14. Composition comprising at least two probes according to claim 12.

15. A kit for inferring the nucleotide sequence at codons of interest in the HIV RT gene and/or the amino acids corresponding to these codons and/or the antiviral drug resistance spectrum of HIV isolates present in a biological sample comprising the following components:

(i) when appropiate, a means for releasing, isolating or concentrating the polynucleic acids present in said sample;

(ii) when appropriate, at least one of the above-defined set of primers;

(iii) at least two of the probes as defined above, possibly fixed to a solid support;

(iv) a hybridization buffer, or components necessary for producing said buffer;

(v) a wash solution, or components necessary for producing said solution;

(vi) when appropriate, a means for detecting the hybrids resulting from the preceding hybridization.

(vii) when appropriate, a means for attaching said probe to a solid support.

16. A kit for inferring the HIV RT resistance spectrum of HIV in a biological sample, coupled to the identification of the HIV isolate involved, comprising the following components:

(i) when appropiate, a means for releasing, isolating or concentrating the polynucleic acids present in the sample;

(ii) when appropriate, at least one of the sets of primers as defined above;

(iii) at least one of the probes as defined above, possibly fixed to a solid support;

(iv) a hybridization buffer, or components necessary for producing said buffer;

(v) a wash solution, or components necessary for producing said solution;

(vi) when appropriate, a means for detecting the hybrids resulting from the preceding hybridization;

(vii) when appropriate, a means for attaching said probe to a solid support.

Figure 1: Natural and drug-induced variability in the vicinity of codons 41, 50, 67-70, 70-75, 151, 181-184, 215 and 219 of the HIV RT gene.

Region I

38	39	40	41	42	43	44
TGT	ACA	GAA	ATG	GAA	AAG	GAA
		G	***T***	G	A	
			C		G	

Region II

47	48	49	50	51	52	53
ATT	TCA	AAA	ATT	GGG	CCT	GAA
		G	***G***			
			C			
			C			

Region III

65	66	67	68	69	70	71	72
AAA	AAA	GAC	AGT	ACT	AAA	TGG	AGA
G	G	***A***		A	***G***		
				A	G		
				GA			
				G			

Region IV

73	74	75	76	77
AAA	TTA	GTA	GAT	TTC
G	***G***	G	C	
		AC		

Region V

148	149	150	151	152	153	154
GTG	CTT	CCA	CAG	GGA	TGG	AAA
	C		***AT***			
	G	G	A			
			T			

Region VI

180	181	182	183	184	185	186	187
ATC	TAT	CAA	TAC	ATG	GAT	GAT	TTA
		G	T	***A***	C	G	GG
				G			G
	G			***G***			

Region VII

212	213	214	215	216
TGG	GGA	TTT	ACC	ACA
	G	C	T	
		A	*TA*	
		C	*TT*	

Region VIII

217	218	219	220
CCA	GAC	AAA	AAA
	T	G	G
		C	
		G	

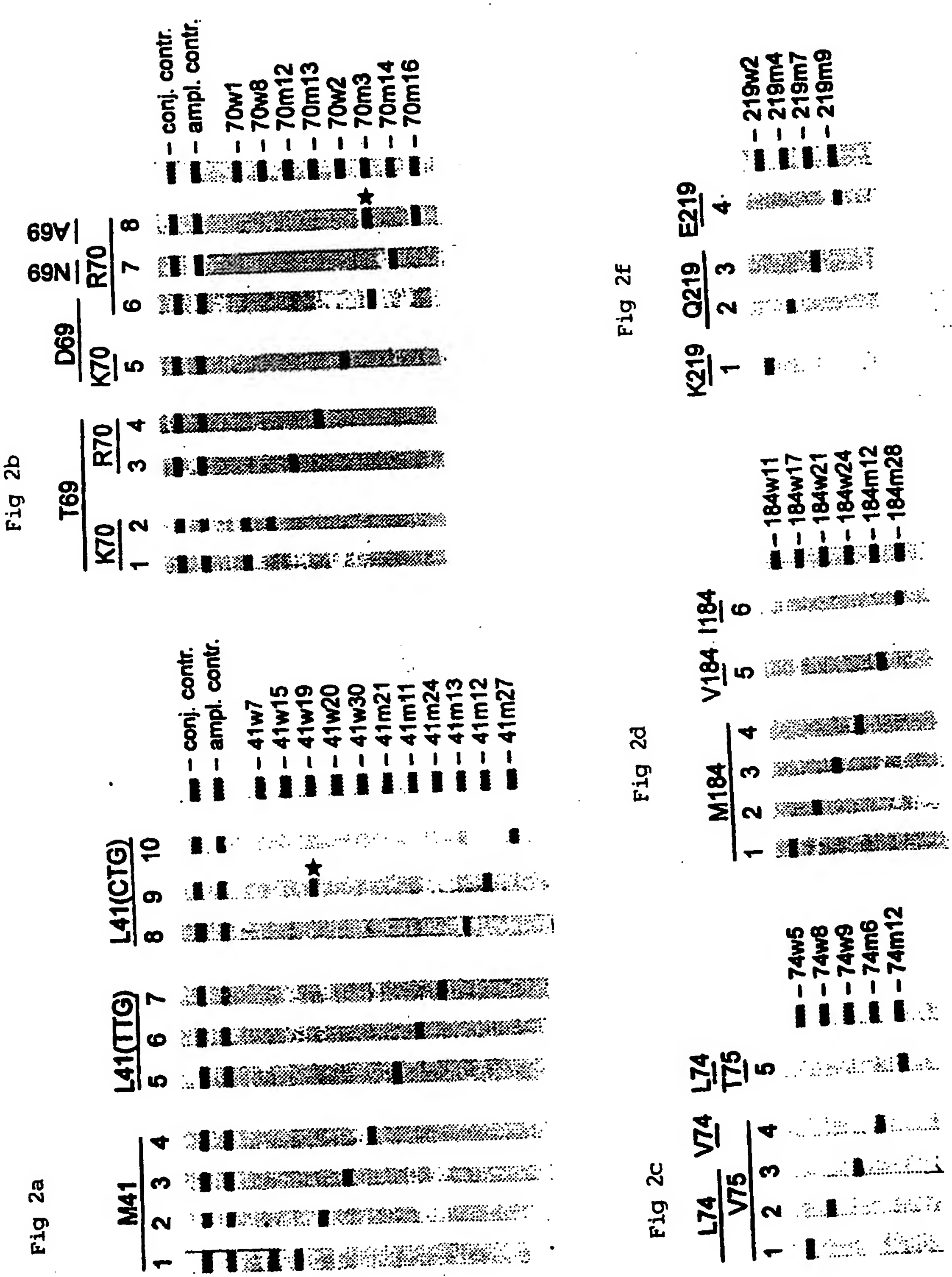

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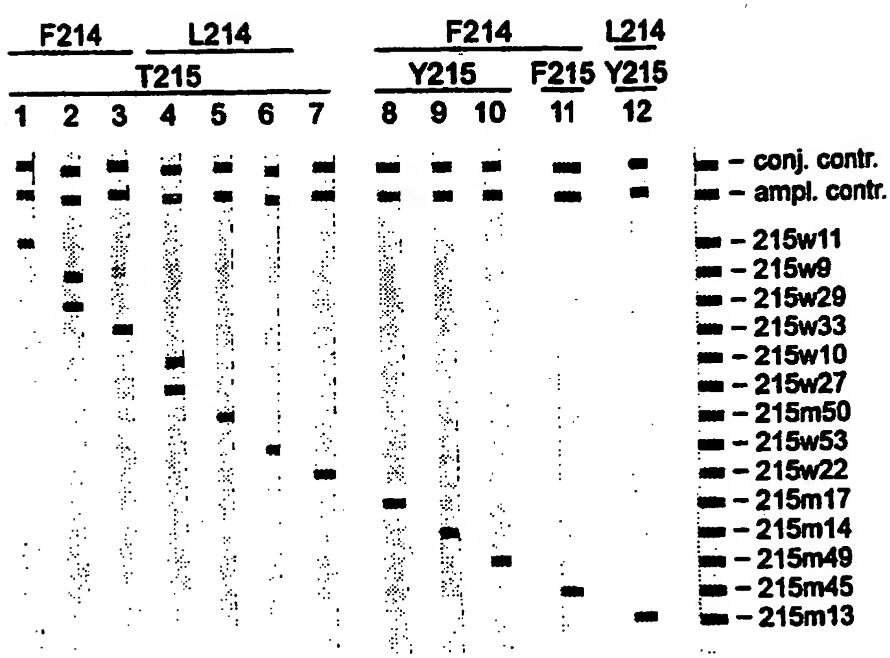
Fig 2e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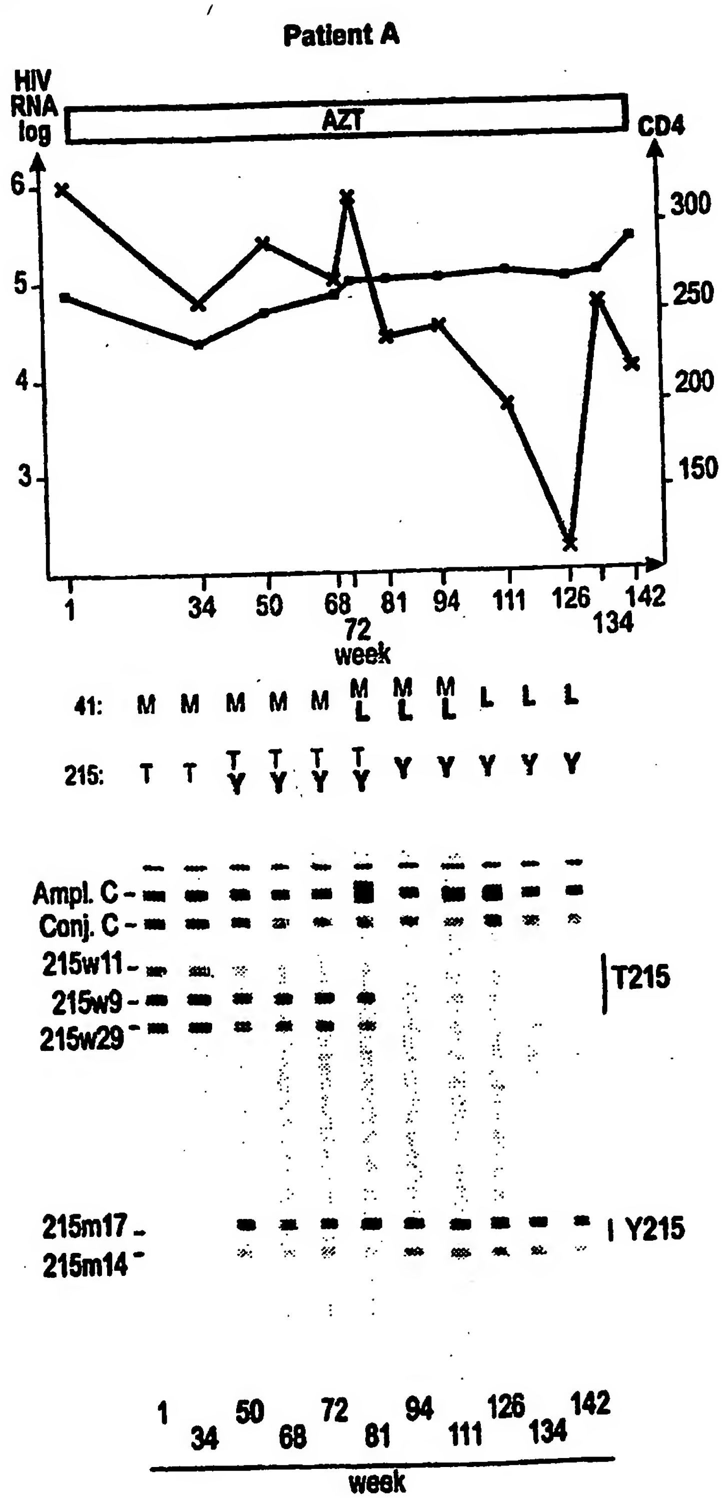
L214
T215
F214
Y215
F215
L214
Y215
1
2
3
4
5
6
7
8
9
10
11
12
– conj. contr.
– ampl. contr.
– 215w11
– 215w9
– 215w29
– 215w33
– 215w10
– 215w27
– 215m50
– 215w53
– 215w22
– 215m17
– 215m14
– 215m49
– 215m45
– 215m13

Figure 3



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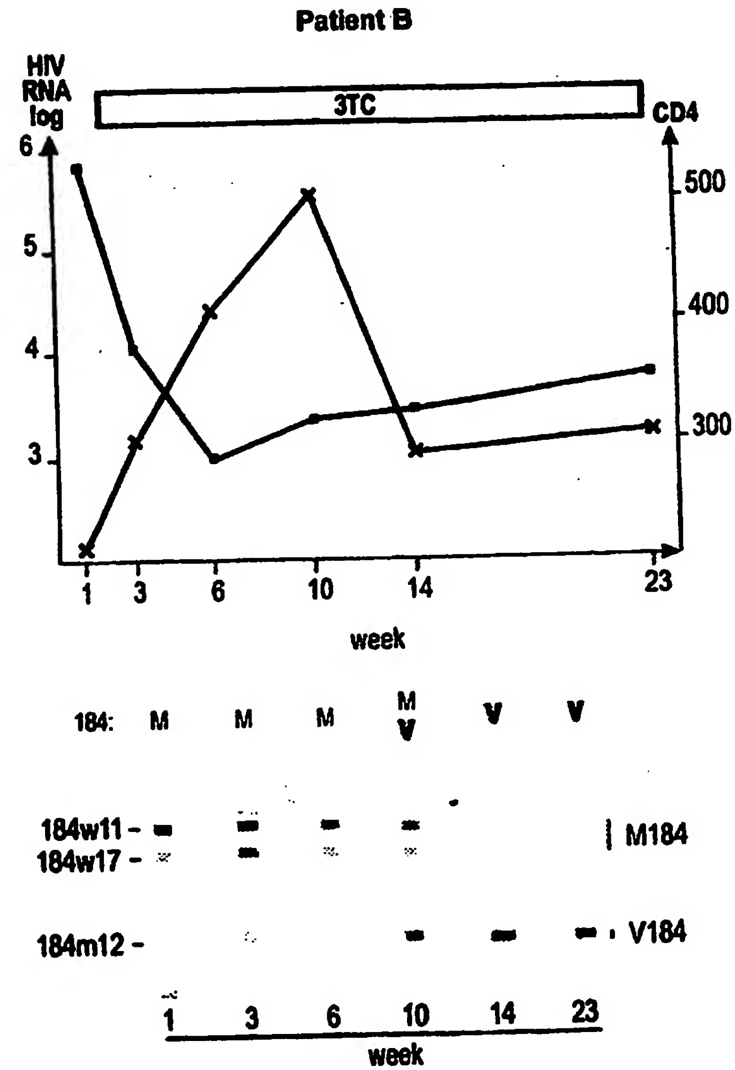

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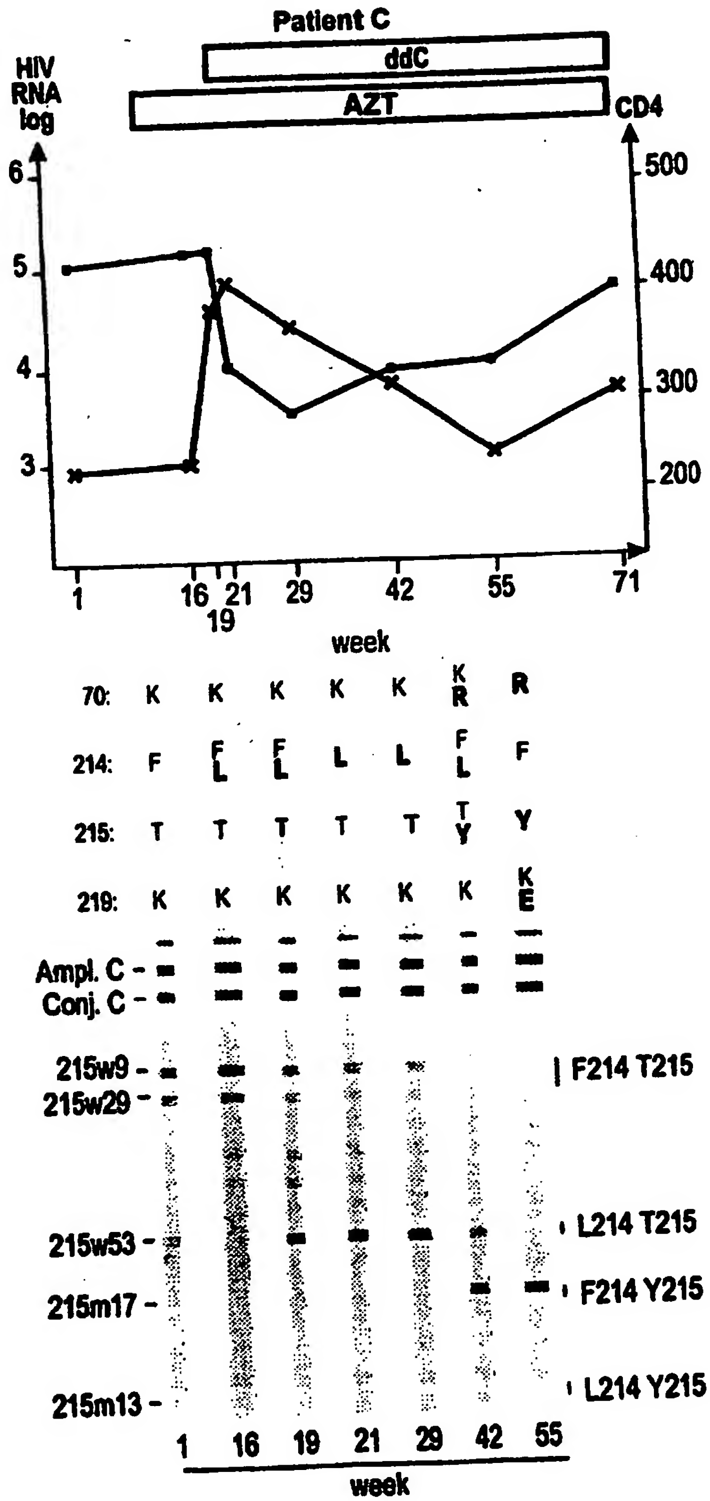
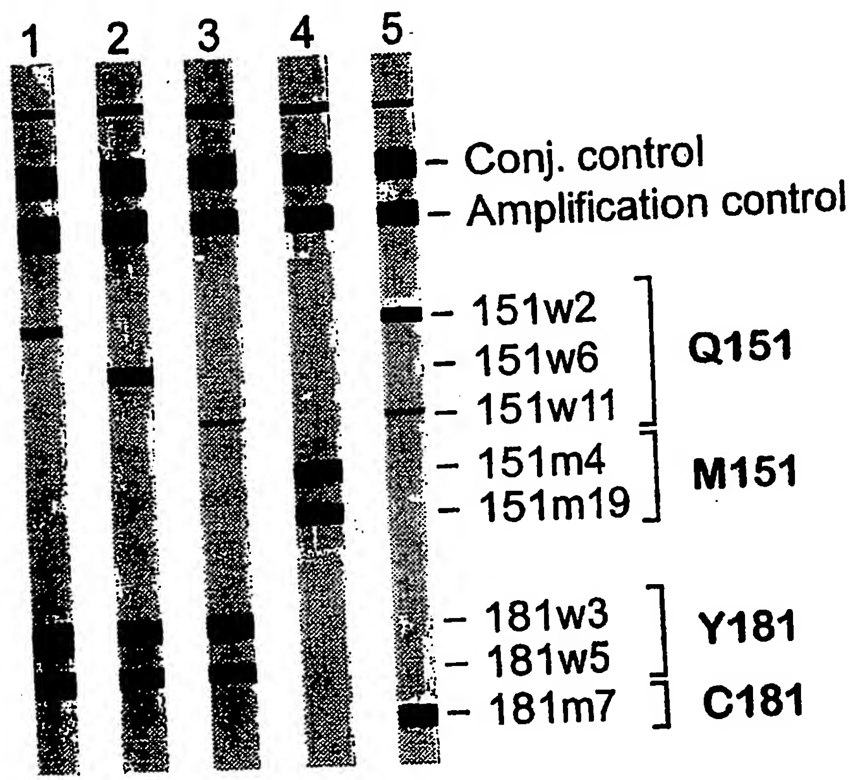

Patient C
ddC
AZT
HIV RNA log
CD4
6
5
4
3
500
400
300
200
1
16
19
21
29
42
55
71
week
70: K K K K K K R R
214: F F L F L L L F L F
215: T T T T T T Y Y
219: K K K K K K K E
Ampl. C
Conj. C
215w9
215w29
215w53
215m17
215m13
F214 T215
L214 T215
F214 Y215
L214 Y215
1 16 19 21 29 42 55
week

Figure 4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2
3
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5
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Amplification control
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151w6
151w11
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151m4
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M151
181w3
181w5
Y181
181m7
C181

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 97/00211

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 92 16180 A (SISKA DIAGNOSTICS INC ;UNIV CALIFORNIA (US)) 1 October 1992 see the whole document	1-3,6, 10-16 4,5,7-9
X Y	EP 0 422 762 A (WELLCOME FOUND) 17 April 1991 see the whole document	1-3,6, 10-16 4,5,7-9
Y	INT ANTIVIRAL NEWS, vol. 3, 1995, page 8-13 XP000614717 MELLORS ET AL.: "Mutations in HIV-1 reverse transcriptase and protease associated with drug resistance" cited in the application see the whole document	4,5,7-9

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
28 May 1997	09.06.97

1

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	INT ANTIVIRAL NEWS, vol. 2, 1994, page 72-74 XP000614718 SCHINAZZI ET AL.: "Mutations in HIV-1 reverse transcriptase and protease associated with drug resistance" cited in the application see the whole document ---	4,5,7-9
Y	SCIENCE, vol. 246, no. 4934, 1 December 1989, pages 1155-1158, XP000371785 LARDER B A ET AL: "MULTIPLE MUTATIONS IN HIV-1 REVERSE TRANSCRIPTASE CONFER HIGH-LEVEL RESISTANCE TO ZIDOVUDINE (AZT)" see the whole document ---	1-16
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, no. 8, 15 April 1992, pages 3241-3245, XP000371883 ERON J J ET AL: "SUSCEPTIBILITY TESTING BY POLYMERASE CHAIN REACTION DNA QUANTITATION: A METHOD TO MEASURE DRUG RESISTANCE OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ISOLATES" see the whole document ---	1-16
Y	THE JOURNAL OF INFECTIOUS DISEASES, vol. 164, no. 6, 1 December 1991, pages 1066-1074, XP000373571 GINGERAS T R ET AL: "USE OF SELF-SUSTAINED SEQUENCE REPLICATION AMPLIFICATION REACTION TO ANALYZE AND DETECT MUTATIONS IN ZIDOVUDINE-RESISTANT HUMAN IMMUNODEFICIENCY VIRUS" see the whole document ---	1-16
Y	WO 94 12670 A (INNOGENETICS NV ;MAERTENS GEERT (BE); STUYVER LIEVEN (BE); ROSSAU) 9 June 1994 cited in the application see the whole document ---	1-16
Y 1	J GEN VIROL, vol. 74, 1993, pages 1093-1102, XP002022992 STUYVER ET AL.: "Typing of HCV isolates and characterisation of new subtypes using a line probe assay" cited in the application see the whole document ---	1-16
1 A	EP 0 532 258 A (JAPAN IMMUNO INC) 17 March 1993 ---	

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOTECHNIQUES, vol. 19, no. 3, 1 September 1995, pages 442-447, XP000541924 LIPSHUTZ R J ET AL: "USING OLIGONUCLEOTIDE PROBE ARRAYS TO ACCESS GENETIC DIVERSITY" ---	1-3
P,X	J. VIROL, vol. 70, February 1996, pages 1086-1090, XP002031823 IVERSEN ET AL.: "Multidrug resistant HIV-1 strains resulting from combination antiretroviral therapy" cited in the application see the whole document -----	

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BNSDOCID: <WO_______9727332A1_I_>

INTERNATIONAL SEARCH REPORT

Information on [illegible] family members

International Application No
PCT/EP 97[illegible]211

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9216180 A	01-10-92	AU 1741692 A	21-10-92
EP 0422762 A	17-04-91	AU 638475 B	01-07-93
		AU 6082890 A	14-02-91
		CA 2022942 A	10-02-91
		DE 69019795 D	06-07-95
		DE 69019795 T	02-11-95
		ES 2072983 T	01-08-95
		IE 69846 B	02-10-96
		JP 3133400 A	06-06-91
		US 5409810 A	25-04-95
WO 9412670 A	09-06-94	AU 5628294 A	22-06-94
		CA 2128528 A	09-06-94
		EP 0637342 A	08-02-95
		JP 7503143 T	06-04-95
EP 0532258 A	17-03-93	CA 2077709 A	10-03-93
		JP 2542994 B	09-10-96
		JP 5301887 A	16-11-93
		US 5427909 A	27-06-95

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Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°./Patent Nr./Patent No./Brevet n°
CMD/FP6117964	01944452.0-1222/US0118882

Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire
Virologic, Inc.

COMMUNICATION

The European Patent Office herewith transmits the partial European search report under Rule 46(1) EPC relating to the above-mentioned European patent application.

Copies of the documents cited in the search report are enclosed.

The applicant's attention is drawn to the following:

The Search Division informs the applicant that if the European search report is also to cover inventions other than the invention first mentioned in the claims, a further search fee must be paid for each of these inventions, within ONE MONTH after notification of this communication.

If the application has been filed up to 30 June 1999, the search fee in force before 01 July 1999 (EUR 869,--) or the equivalent applicable on the date of payment is payable.
This applies also to the search fees requested under Rule 46(1) EPC.
See also OJ EPO 06/1999, 405.

☐ The abstract was modified by the Search Division and the definitive text is attached to the present communication.

☒ Additional set(s) of copies of the documents cited in the European search report is (are) enclosed as well.

Note to users of the automatic debiting procedure:

Unless the EPO receives prior instructions to the contrary, the search fee(s) will be debited on the last day of the period for payment. For further details see the Arrangements for the automatic debiting procedure, Supplement to OJ EPO 02/1999.

REGISTERED LETTER

European Patent Office

SUPPLEMENTARY PARTIAL EUROPEAN SEARCH REPORT

under Rule 45, paragraph 1 of the European Patent Convention

Application Number EP 01 94 4452

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	WO 99/61658 A (VIROLOGIC INC) 2 December 1999 (1999-12-02) * claims 1-72 *	1-6	C12Q1/70
Y	WO 97/27332 A (INNOGENETICS NV ; STUYVER LIEVEN (BE); LOUWAGIE JOOST (BE); ROSSAU RUD) 31 July 1997 (1997-07-31) * page 4, line 15 - page 5, line 11; table 2 *	1-6	
Y	CLERCQ DE E: "DEVELOPMENT OF RESISTANCE OF HUMAN IMMUNODEFICIENCY VIRUS (HIV) TO ANTI-HIV AGENTS: HOW TO PREVENT THE PROBLEM?" INTERNATIONAL JOURNAL OF ANTIMICROBIAL AGENTS, AMSTERDAM, NL, vol. 9, no. 1, 1997, pages 21-36, XP000878561 ISSN: 0924-8579 * the whole document *	1-6	
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TECHNICAL FIELDS SEARCHED (Int.Cl.7)

C12Q

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

see sheet B

The present partial European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims.

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Place of search	Date of completion of the search	Examiner
The Hague	27 October 2004	Schmitt, A

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C13)

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 01 94 4452

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Y	SALOMON H ET AL: "Prevalence of HIV-1 resistant to antiretroviral drugs in 81 individuals newly infected by sexual contact or injecting drug use. Investigators of the Quebec Primary Infection Study." AIDS (LONDON, ENGLAND) 28 JAN 2000, vol. 14, no. 2, 28 January 2000 (2000-01-28), pages F17-F23, XP008037438 ISSN: 0269-9370 * the whole document * -----	1-6	
Y	MELLORS J W ET AL: "MUTATIONS IN HIV-1 REVERSE TRANSCRIPTASE AND PROTEASE ASSOCIATED WITH DRUG RESISTANCE" INTERNATIONAL ANTIVIRAL NEWS, CHURCHILL LIVINGSTONE, EDINBURGH, GB, vol. 3, 1995, pages 8-13, XP000614717 ISSN: 0965-2310 * the whole document * -----	1-6	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
A	KANKI P J ET AL: "VIROLOGY OF HIV-1 AND HIV-2: IMPLICATIONS FOR AFRICA" AIDS, LONDON, GB, vol. 11, no. SUPPL B, 1997, pages S33-S42, XP008035289 ISSN: 0269-9370 * the whole document * -----	1-6	

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EPO FORM 1503 03.82 (P04C10)

European Patent Office

LACK OF UNITY OF INVENTION SHEET B

Application Number
EP 01 94 4452

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

1. claims: 1-6 (partially)

   Method for assessing the effectiveness of non-nucleoside reverse transcriptase antiretroviral therapy of an HIV-infected patient comprising evaluating whether a patient's plasma sample contains nucleic acid encoding HIV reverse transcriptase (HIV-RT) having a mutation at codon 230 or at codons 230 and 103, or at codons 230 and 181; and method and resistance test vector for assessing the biological effectiveness of a candidate HIV antiretroviral drug compound comprising a patient-derived segment 'of reverse transcriptase! which comprises a mutation at said codon(s).

   ---

2. claims: 1-6 (partially)

   Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230 and 101.

   ---

3. claims: 1-6 (partially)

   Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230 and 190.

   ---

4. claims: 1-6 (partially)

   Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230 and 221.

   ---

5. claims: 1-6 (partially)

   Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230 and 238.

   ---

6. claims: 1-6 (partially)

   Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 101, 103.

   ---

7. claims: 1-6 (partially)

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 101, 190.

---

8. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 101, 221.

---

9. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 101, 238.

---

10. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 103, 190.

---

11. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 103, 221.

---

12. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 103, 238.

---

13. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 190, 221.

---

14. claims: 1-6 (partially)

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 190, 238.

---

15. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 221, 238.

---

16. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 101, 103, 190.

---

17. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 101, 103, 221.

---

18. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 101, 103, 238.

---

19. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 101, 190, 221.

---

20. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 101, 190, 238.

---

21. claims: 1-6 (partially)

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 101, 221, 238.

---

22. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 103, 190, 221.

---

23. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 103, 190, 238.

---

24. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 103, 221, 238.

---

25. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 190, 221, 238.

---

26. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 101, 103, 190, 221.

---

27. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 101, 103, 190, 238.

---

28. claims: 1-6 (partially)

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 101, 103, 221, 238.

29\. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 101, 190, 221, 238.

30\. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 103, 190, 221, 238.

31\. claims: 1-6 (partially)

Methods and resistance test vector as described in the previous invention, wherein the HIV-RT has mutations at codons 230, 101, 103, 190, 221, 238.

32\. claims: 1-6 (partially)

Method for assessing the effectiveness of non-nucleoside reverse transcriptase antiretroviral therapy of an HIV-infected patient comprising evaluating whether a patient's plasma sample contains nucleic acid encoding HIV-RT having a mutation at codon 241 or at codon 241 and further codons; and method and resistance test vector for assessing the biological effectiveness of a candidate HIV antiretroviral drug compound comprising a patient-derived segment 'of reverse transcriptase! which comprises a mutation at codon 241 or at codon 241 and further codons.

33\. claims: 1-6 (partially)

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

Method for assessing the effectiveness of non-nucleoside reverse transcriptase antiretroviral therapy of an HIV-infected patient comprising evaluating whether a patient's plasma sample contains nucleic acid encoding HIV-RT having a mutation at codon 245 or at codon 245 and further codons; and method and resistance test vector for assessing the biological effectiveness of a candidate HIV antiretroviral drug compound comprising a patient-derived segment 'of reverse transcriptase! which comprises a mutation at codon 245 or at codon 245 and further codons.

---

34. claims: 1-6 (partially)

Method for assessing the effectiveness of non-nucleoside reverse transcriptase antiretroviral therapy of an HIV-infected patient comprising evaluating whether a patient's plasma sample contains nucleic acid encoding HIV-RT having a mutation at codon 270 or at codon 270 and further codons; and method and resistance test vector for assessing the biological effectiveness of a candidate HIV antiretroviral drug compound comprising a patient-derived segment 'of reverse transcriptase! which comprises a mutation at codon 270 or at codon 270 and further codons.

The Applicant is to note that if the Applicant pays additional fees for one (or more) not yet searched group(s) of invention(s) as defined above, then the further search(es) may reveal further prior art that gives evidence of a further lack of unity 'a posteriori' within one (or more) of the not yet searched group(s).

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO.

EP 01 94 4452

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
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27-10-2004

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9961658	A	02-12-1999	AU	772511	B2	29-04-2004
			AU	4207599	A	13-12-1999
			BR	9911600	A	13-02-2001
			CA	2329140	A1	02-12-1999
			CN	1311823	T	05-09-2001
			EP	1082454	A1	14-03-2001
			JP	2002516119	T	04-06-2002
			MX	PA00011623	A	17-10-2002
			NZ	508834	A	25-06-2004
			WO	9961658	A1	02-12-1999
WO 9727332	A	31-07-1997	AU	719691	B2	18-05-2000
			AU	1444397	A	20-08-1997
			BR	9704637	A	09-06-1998
			CA	2215073	A1	31-07-1997
			WO	9727332	A1	31-07-1997
			EP	0817866	A1	14-01-1998
			JP	11502727	T	09-03-1999
			US	6331389	B1	18-12-2001
			US	6087093	A	11-07-2000
			US	2003077575	A1	24-04-2003

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